ABSTRACT

Nowadays it is increasingly accepted that interactions with environmental factors must play an important role in determining bone quality and that these interactions must be influenced by genetics. Nevertheless, to date, only few animal studies explore an underlying genetic basis for extrinsic factors effect such as fluoride (F-) effect on bone metabolism.

This study assessed the effect of increasing F- doses on the bone properties in three inbred mouse strains that demonstrate different susceptibilities to developing enamel fluorosis (A/J a "susceptible" strain, 129P3/J a "resistant" strain and SWR/J an "intermediate" strain). Fluoride concentrations were determined in femora and vertebral bodies. Bone mineral density was evaluating through DXA (Dual energy X-ray Absorptiometry). Three-point bend testing of femora, compression testing of vertebral bodies and femoral neck-fracture testing were performed to evaluate mechanical properties. Bone microarchitecture was quantified with microcomputed tomography and strut analysis. Bone formation was evaluated by static histomorphometry. Bone mineralization was quantified with backscattered electron (BSE) imaging and powder x-ray diffraction. Microhardness measurements were taken from the vertebral bodies (cortical and trabecular bone) and the cortex of the distal femur.

Increasing doses of F- had different effects on the bone properties in the three inbred mouse strains. Although significant increases of bone F- concentration could be demonstrated, and no significant effects on bone macroarchitecture and BMD were found in the three strains, mechanical testing showed significant degradation of bone mechanical properties in the "susceptible" A/J strain, whereas moderate degradation in the "intermediate" SWR/J strain and no effect in the "resistant" 129P3/J strain were observed. Evaluation of the structural and material properties of the bone in the three strains showed that this different effect of F- on bone mechanical properties could be explained by an alteration of the mineral-organic interfacial bonding and/or bone matrix proteins, interfering with bone crystal growth inhibition on the crystallite faces as well as bonding between the mineral and organic interface. The smaller bone crystallites of the 129P3/J (resistant) strain may indicate a stronger organic-inorganic interface, reducing crystallite growth rate and increasing interfacial mechanical strength.

MOUSNY MARYLINE

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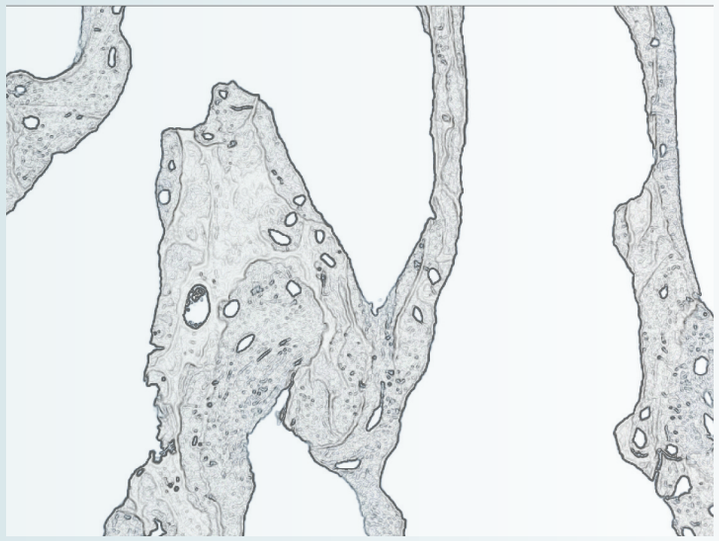

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FACULTÉ DE MÉDECINE

Orthopaedic Research Laboratory

GENETICS DETERMINE BONE SUSCEPTIBILITY TO FLUORIDE

Maryline Mousny

Thesis submitted in conformity with the requirements for the degree of "Docteur en Sciences Médicales"

A mes parents
A mon frère
A Geneviève

Remerciements

Il faut écrire au courant de la plume ; sans chercher les mots.

Jean-Paul Sartre

J'ai respecté ce principe en écrivant ces remerciements. L'évocation de chaque personne m'ayant accompagnée dans ce travail de thèse a été source de pensées que j'ai voulu immortaliser dans ces quelques lignes. J'espère que ces mots seront à la hauteur de mes sentiments.

A raconter ses maux, souvent on les soulage.

Corneille

Je remercie ma famille et mes amis pour leur amour, leur soutien et leurs encouragements. Ils ont su m'écouter sans me juger, me conseiller sans imposer. Leur présence m'a apporté apaisement et réconfort dans les moments difficiles. Ils ont su transformer les moments de découragement et de doutes en nouveaux départs.

L'enthousiasme est un feu dévorant
Qui se dévore lui-même
S'il n'est pas nourri de l'extérieur

Makram Ubeid

Une réponse, c'est forcément le chemin qu'on a déjà parcouru. Seules les questions peuvent montrer le chemin qu'il reste à faire.

Jostein Gaarder

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Quand on voyage vers un objectif, il est très important de prêter attention au chemin. C'est toujours le chemin qui nous enseigne la meilleure façon d'y parvenir et il nous enrichit à mesure que nous le parcourons.

Paulo Coelho

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Il est bien de donner quand on est sollicité, mais il est mieux encore de pouvoir tout offrir à qui n'a rien demandé.[…] L'homme donne peu quand il ne dispose que des biens matériels qu'il possède, mais il donne beaucoup quand il s'offre lui-même.

Khalil Gibran

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Les gens sollicitent vos critiques, mais ils ne désirent seulement que des louanges.

W. Somerset Maugham

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Tu donnes à qui tu veux ce que tu veux.

Alî Abû Hayyân al-Tawhîdî

Nous ne devons rien à personne, nous devons tout à tout le monde.

Khalil Gibran

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMDD	Bone Mineralization Density Distribution
BMU	Basic Metabolic Unit
BV/TV	Trabecular Bone Volume
BVF	Bone Volume Fraction
BSE	Backscattered Electron
BSP	Bone Sialoprotein
Ca^{2+}	Calcium
CaF_2	Calcium Fluoride
% Ca	Calcium Percentages
DXA	Dual energy X-ray Absorptiometry
F	Femur/Femora
F^-	Fluoride
[F]	Fluoride Concentration
FHA	Fluorohydroxyapatite
FN	Femoral Neck
FWHM	Full Width at Half the Maximum Height
HA	Hydroxyapatite
INAA	Instrumental Neutron Activation Analysis
μCT	Microcomputed Tomography
MdV/TV	Mineralized Trabecular Bone Volume
NaF	Sodium Fluoride
NCPs	Noncollagenous organic Proteins
OS/BS	Osteoid Surface
OTh	Osteoid Thickness
OV/BV	Osteoid Volume
%Ca	Calcium Percentages
PMMA	Polymethylmethacrylate
QTLs	Quantitative Trait Loci
SA	Bone Surface Area
SD	Standard Deviation
SMI	Structure Model Index
TbN	Trabecular Number
TbSp	Trabecular Separation
TbTh	Trabecular Thickness
VB	Vertebral body/bodies

CHAPTER 1: INTRODUCTION

1.1. Bone Biology

In order to understand the mechanical properties of bone and the reasons of their alterations with environmental factors, it is necessary to have a general knowledge of bone biology. Therefore we have to consider the full complexity of the hierarchical architecture of bone, from the macro- to the micro-scale, and the influence of the various constituents on bone mechanical properties.

1.1.1 Composition and Structure of Bone

Bone is a natural composite material. By weight, bone contains approximately 60% mineral, 10% water, and about 30% collagenous matrix [1]. Bone exhibits a complex structure with several hierarchical levels ranging from the macro- down to the nanoscale [2, 3, 4, 5]. The different hierarchical levels of bone are summarized in table 1 and Figure 1. All the bone components display an irregular arrangement and orientation, making the material of bone heterogeneous and anisotropic.

Levels	Structures
Macrostructure	• Trabecular (or cancellous) bone • Cortical (or compact) bone
Microstructure (from 10 to 500 µm)	• Haversian systems • Osteons • Single trabeculae
Sub-microstructure (1-10 µm)	• Lamellae
Nanostructure (from a few hundred nanometers to 1 µm)	• Fibrillar collagen • Embedded mineral
Sub-nanostructure (below a few hundred nanometers)	• Molecular structure of constituent elements such as mineral, collagen, and non-collagenous organic proteins

Table 1: Hierarchical structural organization of bone [3].

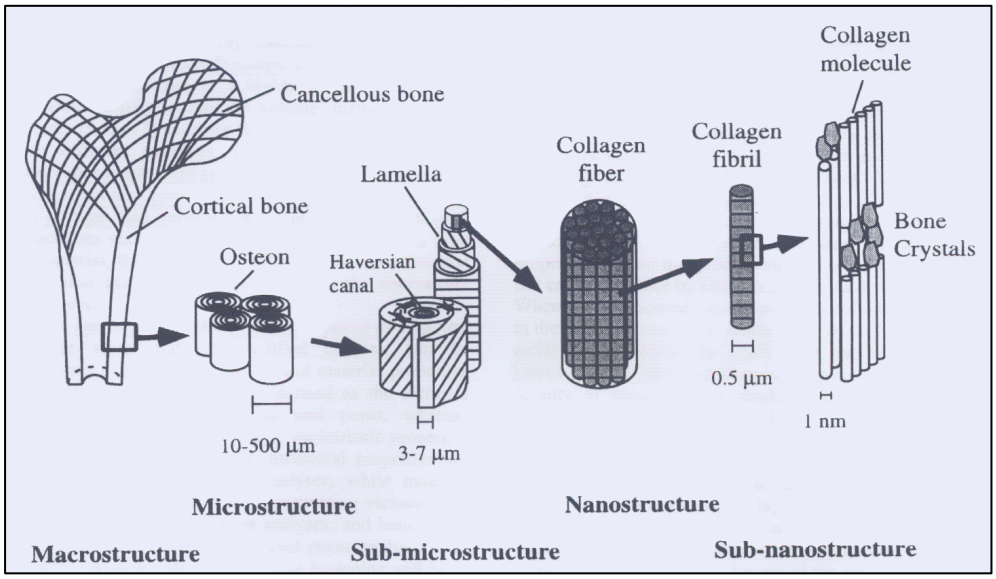

Figure 1: Hierarchical structural organization of bone ranging from the macro- down to the nanoscale, with (a) cortical and trabecular bone; (b) osteons with Haversian systems; (c) lamellae; (d) collagen fiber assemblies of collagen fibrils; (e) bone mineral crystals, collagen molecules and non-collagenous proteins [3].

Bone macrostructure

At the macrostructure level, bone is described as cortical (or compact) or trabecular (or cancellous, or spongy) (Figure 2). Although both types of bone are most easily distinguished by their degree of porosity or density, reliable differentiation can only be achieved by histological evaluation [3]. Cortical bone, which is rigid and dense, is found mainly in the middle shaft of long bones or shells of other bones. Trabecular bone has a highly porous structure with bony trabecular interconnected struts and marrow-filled cavities. It is found predominantly in the epiphysis, ribs and spine. In an intermediate form, differentiation between the two types of bone is difficult. This tissue consists of cortical bone wrapped around older trabecular bone and has irregular, sinuous convolutions of lamellae [6].

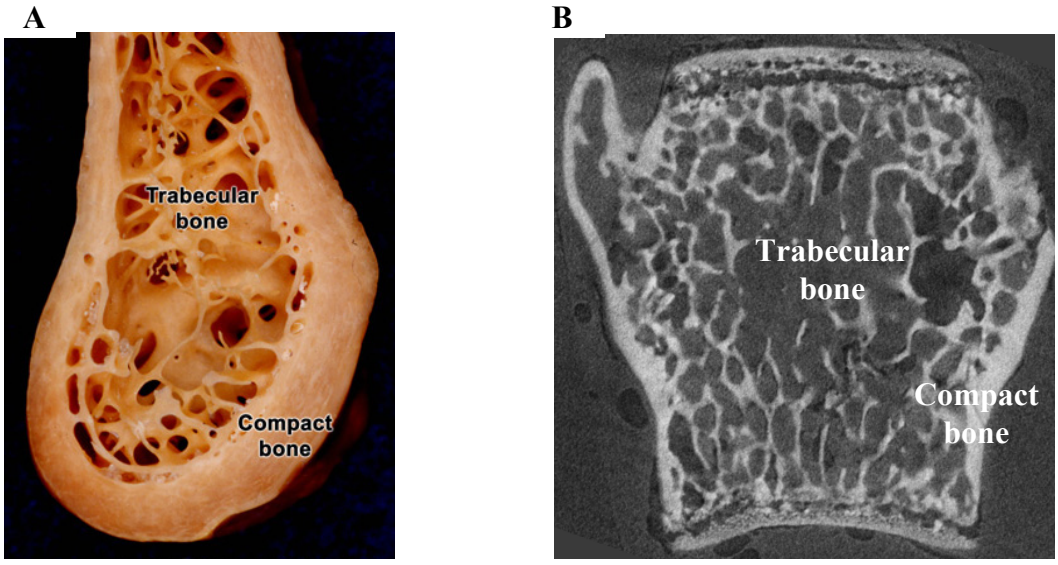

Figure 2: **A.** Body of the mandible [7]. **B.** Coronal section of a thoracic vertebral body, obtained with microcomputed tomography.
The compact bone (outer layer) can be distinguished from the trabecular bone (inner spongy structure).

Bone microstructure

At the microstructure level, osteons (or Haversian systems) are found in cortical bone and trabeculae in trabecular bone [6]. Mineralized collagen fibers form into planar arrangements called lamellae. In cortical bone, these sheets (lamellae) wrap in concentric layers around a central canal to form what is called an *osteon* or a *Haversian system* (Figure 3). These concentric osteonal lamellae have fibre orientations alternating to each other. The osteon looks like a cylinder about 200-250 μm in diameter running roughly parallel to the long axis of the bone [3] (Figure 4).

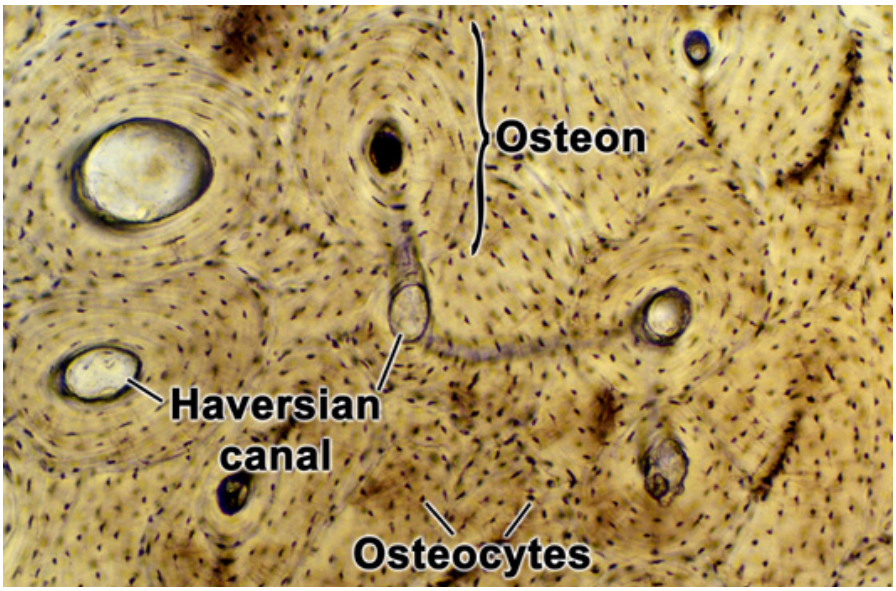

Figure 3: In cortical bone, concentric lamellae wrap around a central Haversian canal to form the so-called osteon [7].

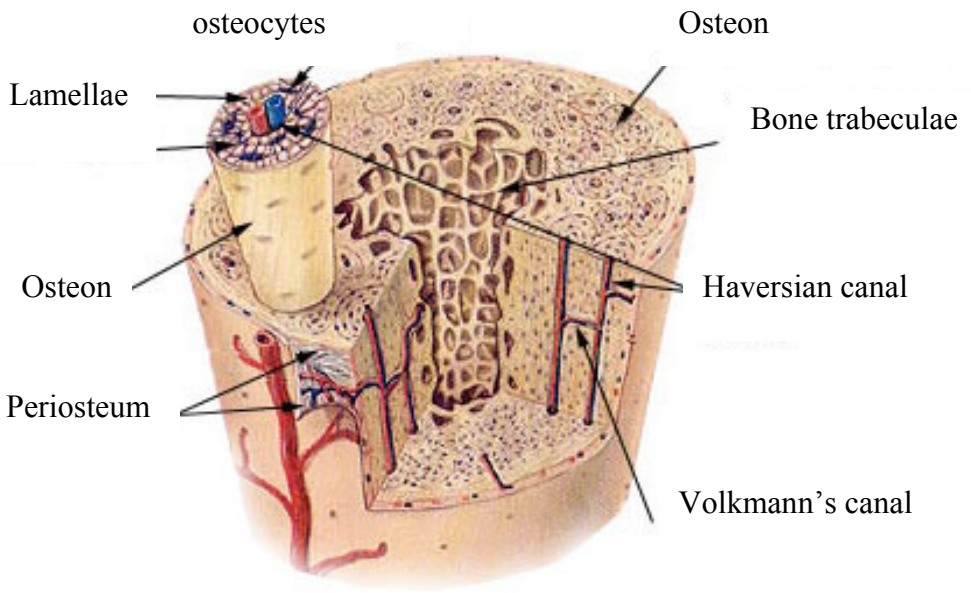

Figure 4: In cortical bone, the osteon looks like a cylinder running roughly parallel to the long axis of the bone. Osteons are connected with transverse canals called Volkmann's canal [8].

Trabecular bone consists of trabeculae (or struts) that are osseous structures having a platelike or rodlike configuration, with a trabecular rod being about 50-300 μm in diameter [3]. Trabecular bone is made of an interconnecting framework of trabeculae in a number of combinations, with the following basic cellular structures: rod-rod, rod-plate, or plate-plate (Figure 5).

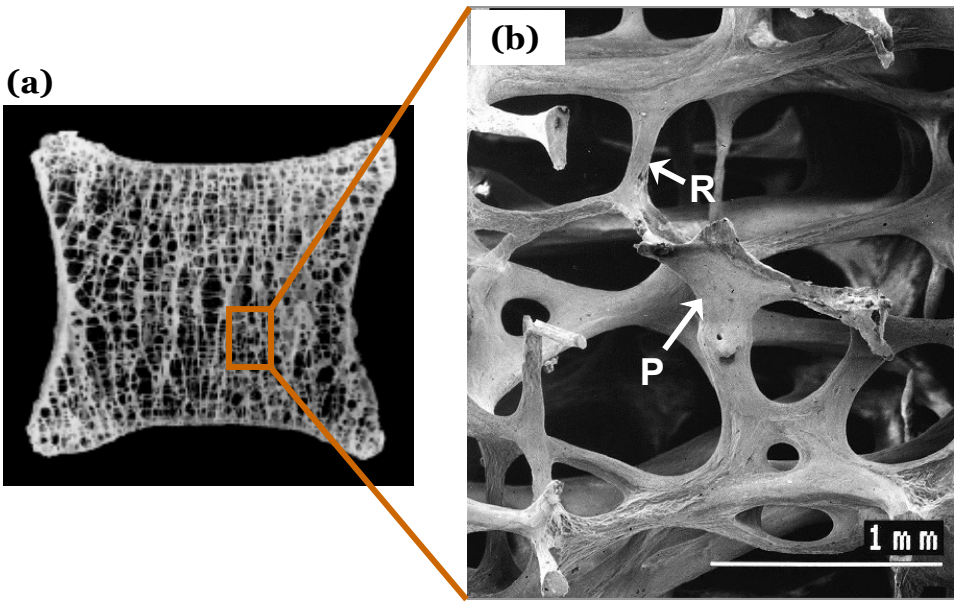

Figure 5: (a) Micrograph of healthy human vertebra [9]

(b) Trabecular bone structure (adapted from [10]). The trabeculae that are osseous structures having a platelike (P) or rodlike (R) configuration, interlace and anastomose to form a spongy structure.

Bone sub-microstructure

At the sub-microstructure level, we find the bone lamellae. The exact arrangement and orientation of the substance of a lamella is not well known. There may be differences in the lamellae encountered in cortical and trabecular bone [3]. It was generally admitted that the collagen fibers in a lamella of an osteon, lied in parallel in each lamella, with a change in the orientation of fibrils from one lamella to the next described as a twisted plywood or helicoidal structure [2, 3, 11]. According to this representation, adjacent lamellae have different orientations: either longitudinal, with the collagen fibers along the long axis of the lamellar sheet, or transverse, with the collagen fibers perpendicular to the long axis. However, another model suggests that the

arrangement of the fibers in a lamella is highly interwoven with no apparent preferred orientation [12]. According to this model, a lamella can therefore no longer be considered as containing individual, highly oriented unbranched collagen bundles. Overall, collagen has a basically parallel orientation but the fibers form a continuum both within a single lamella and between lamellae [3].

Bone nanostructure and sub-nanostructure

At the nanostructural and sub-nanostructural levels, bone consists of:

- type I collagen fibrils which accounts for approximately 90% of the whole organic matrix
- mineral crystals of hydroxyapatite (calcium phosphate) [2, 3]
- water [13]
- several noncollagenous organic proteins (NCPs) [14, 15], including phosphoproteins such as osteopontin, sialoprotein, osteonectin, and osteocalcin.
- proteoglycans and phospholipids.

Type I collagen is a large fibrous protein with a highly repetitive sequence [Gly (glycine)-X-Y]$_n$ (often X is proline and Y is hydroxyproline). This repetitive sequence allows three polypeptide chains (called α chains) to fold into a unique triple-helical structure. Bone collagen molecules are triple helical molecules that are composed of two α_1 polypeptide chains and one α_2 chain [2, 16]. Collagen molecules secreted by osteoblasts self-assemble into fibrils with a specific tertiary structure having a 67 nm periodicity and 40 nm gaps or holes between the ends of the molecules (Figures 6 and 7).

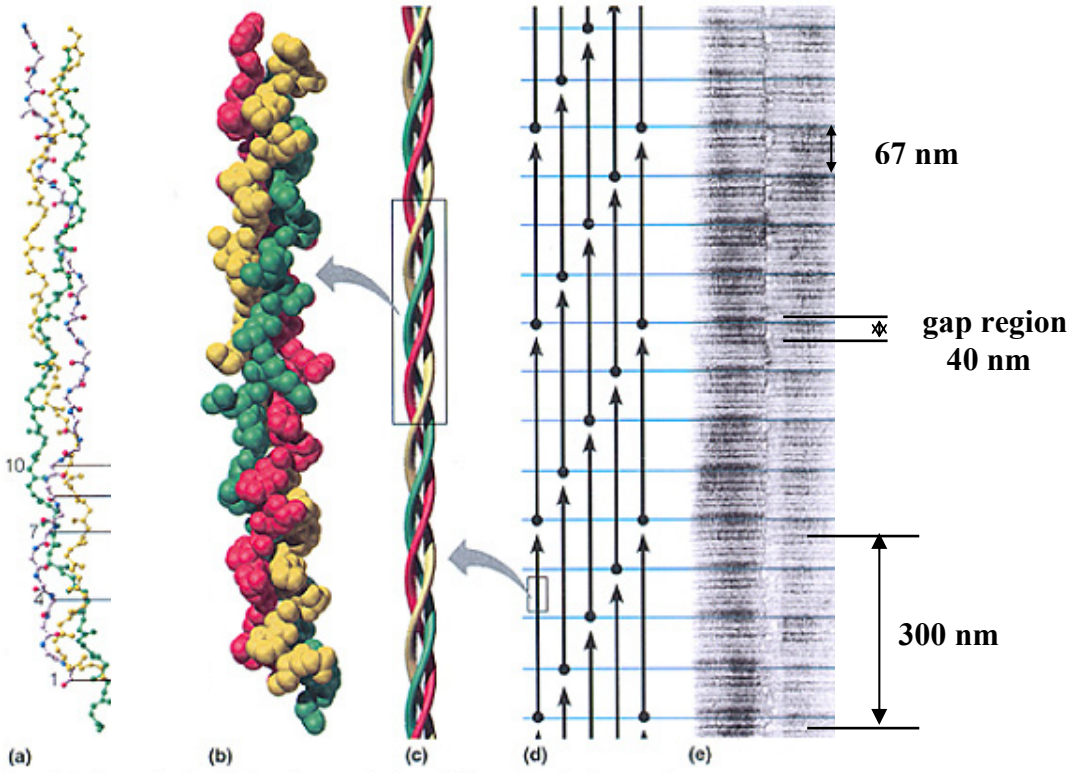

Figure 6: Hierarchical structure of collagen [adapted from 17]

(a-b-c) Type I collagen is a large fibrous protein made up of three polypeptide chains.

(d) Collagen molecules assembled into fibrils with a specific tertiary structure having a 67 nm periodicity.

(e) Banded aspect displayed by type I collagen fibrils in electron microscopy.

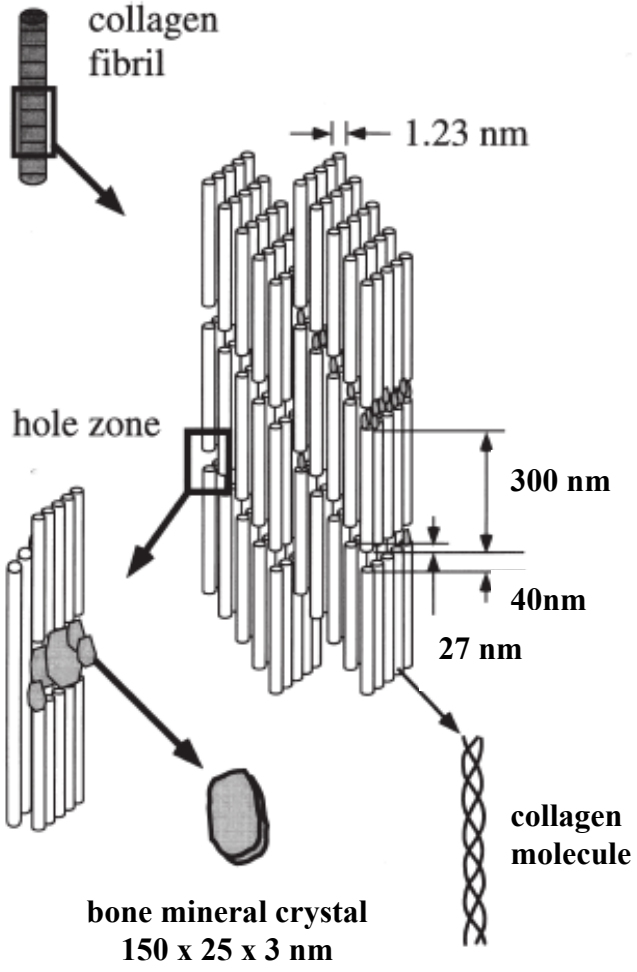

Figure 7: Hierarchical structure of collagen molecules and fibrils [adapted from 3].

In fibrils, molecules of collagen are parallel to each other; they overlap each other by multiples of 67 nm, with each molecule being 300 nm long. The triple helical molecules are arranged in a staggered array such that a 40 nm gap or hole exists between the NH_2-terminus of one triple helical molecule and the COOH-terminus of the next molecule [2, 3, 6]. This quarter-staggered assembly explains the banded aspect displayed by type I collagen fibrils in electron microscopy. The molecules are stabilized by intra- and intermolecular cross-links [18]. The collagen cross-link profile has been suggested to correlate with the structural organization of the trabeculae in human vertebral bodies [19]. The collagen fibrils are almost always present as bundles (to form collagen fibers) or aligned arrays, and these can be arranged in a variety of different motifs. One such motif is the lamellar structure. The collagen acts as a framework in which plate-shaped crystals of hydroxyapatite form [20]. In addition to having structural roles, collagen also has direct effects on important bone cell functions including apoptosis, cell proliferation and differentiation [15].

Bone crystallites are very small, plate-shaped crystals whose thickness ranges from 2 to 7 nm, length from 150 to 200 nm and width from 10 to 80 nm [21]. It must be noted that, although most studies describe the mineral particle as plate-like in shape [2], there is still an ongoing debate about their real shape, needles *versus* platelets. Nowadays, it is recognized that the needle-like shape of the mineral crystals might be due to the side-on view of the particles with transmission electron microscopy [21]. However the existence of more needle-like mineral particles in bone tissues of certain species or developmental ages cannot be excluded [21].

It is generally accepted that the apatite structure is a prototype for the structure of bone mineral. Hydroxyapatite has a unit cell represented by the formula: $Ca_{10}(PO_4)_6(OH)_2$. Bone mineral is thus structurally related to the naturally occurring geologic mineral,

hydroxyapatite (HA), but differs in crystal size, perfection and the nature of included vacancies and impurities (Mg^{+2}, Sr^{+2}, CO_3^{-2}, HPO_4^{-2}, etc) (Figure 8) [22, 23]. Bone mineral contains a substantial amount of carbonate ions (5-8 wt%), believed to substitute mainly in phosphate ion lattice positions [24]. The bone apatite contains small but significant amounts of impurities such as HPO_4, Na, Mg, citrate, carbonate, K, zinc and others [3, 6] and is deficient in calcium ion, containing simple calcium vacancies [13]. The high levels of substitutions and defects in bone mineral crystallites are believed to help limit crystal growth [13].

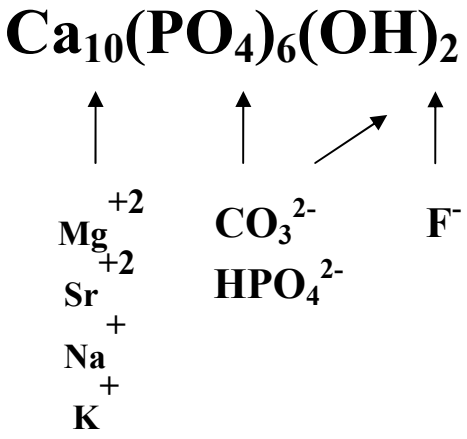

Figure 8: Chemical formula for apatite and lattice location for some impurities found in bone mineral [22].

Bone mineral is a poorly crystalline hydroxyapatite phase. Ion substitutions are abundant. For example, Na^+ and Mg^{+2} are substituting Ca^{+2} ions, HPO_4^{2-} ions substituting the phosphate ions, F^- substituting OH^- and CO_3^{2-} substituting for either phosphate or hydroxyl groups. Since it is bathed in aqueous biological fluids, the type and extent of these substitutions change with time, resulting in modifications of the mineral maturity.

Apatite crystals occur within the discrete spaces within the collagen fibrils (Figure 7) [21]. Early mineralization is initially intrafibrillar (inside the fibers) and then interfibrillar (between the fibers) [3, 4]. The degree of extra- versus intrafibrillar mineral is still a matter of debate. Some authors consider the entire composite to be a "foam" of mineral inside a collagen matrix [25, 26], which blurs the distinction between extra- and intrafibrillar mineral. The process of bone mineral deposition begins with the nucleation of HA crystals at multiple sites on the collagen fibrils [22]. Thanks to their affinity for HA, extracellular matrix proteins may act as nucleators and also regulate the way the crystals grow, hence determining their size and shape (Figure 9). The mineral crystals grow with a specific crystalline orientation: the *c* axes of the crystals are roughly parallel to the long axes of the collagen fibrils [3, 27]. The crystals are arranged in layers that traverse the fibril [28].

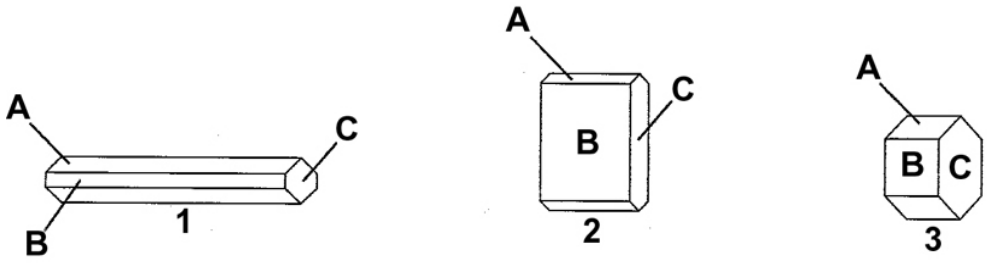

Figure 9: Crystal shape may be determined by selective inhibition of growth on differing faces. Inhibition of growth on the A and B faces produces a needle-shaped crystal (1). Inhibition of the B face alone produces a plate-shaped crystal (2). No inhibition produces a block-shaped crystal (3). Noncollagenous proteins may bind selectively to different surfaces of the crystal, preventing further growth and thereby determining its final size and shape [7].

Bone mineralization has been described as occurring in two phases [29, 30, 31]: the primary mineralization, when bone mineral is deposited quickly to reach 65-70% of final mineralization and the secondary mineralization, when the initial crystals mature to their final size (the remainder of the mineral is gradually deposited over a period of years [32]). Crystals increase in size by the addition of ions to the smallest crystals, by secondary nucleation, and by fusion or agglomeration of existing crystals [22, 33]. During secondary nucleation, each crystal can serve as a branch point for nucleation of additional crystals. A multitude of extrinsic (diet, health status, treatments) and intrinsic (collagen properties, matrix proteins distribution, age, rates of bone turnover, diseases) factors influence the size and composition of bone mineral crystals [22]. Crystals from newly formed bone are generally smaller in size than mature bone crystals [34]. Bone mineralization is influenced by the rate of bone turnover and has been shown to change with age [29, 32, 35]. The bone matrix is not uniformly mineralized and shows important local variations [21]. The bone remodeling together with the time course of mineralization of newly formed bone matrix generate a characteristic Bone Mineralization Density Distribution (BMDD). Therefore the bone material is composed of bone packets, each having its own mineral content corresponding to its tissue age. BMDD, a parameter reflecting the age and size distribution of the bone packets within the tissue, has been shown to be nearly constant in healthy adults independent of age, gender, ethnic origin and skeletal site [36, 37].

Water is an essential component of bone, accounting for up to 25% by weight [13]. Much of this water occurs in pore spaces responsible for nutrient diffusion and contributing to the viscoelastic properties of the material. With bone aging, the increase in mineral content occurs at the expense of the water content [4].

Noncollagenous organic proteins (NCPs) are located between mineralized collagen fibrils [38] and are known to be implicated in bone mineralization [39, 40, 41, 42]. Several of these are plasma proteins (albumin, transferin, IgG, 2-HS glycoprotein), which have been sequestered by the mineralized phases, while others are bone specific proteins, synthesized by bone cells [43]. There are more than 200 NCPs, but together they generally represent less than 10% of the total bone protein content [4]. The majority of the NCPs are anionic, with negatively charged groups that can bind Ca^{2+} in solution and on the surface of hydroxyapatite [39]. The most abundant components of NCPs are osteonectin and osteocalcin (also called bone Gla protein). Osteonectin represents 23% of the NCPs and has a high affinity for calcium, hydroxyapatite and type I collagen [43]. Osteocalcin represents 10-20% of the total NCPs in bone. It is synthesized exclusively in the osteoblasts and binds both to hydroxyapatite and to calcium [43]. Bone sialoprotein (BSP) is a NCP that is expressed almost exclusively in mineralized tissues and that has been shown to be a potent nucleator of hydroxyapatite formation *in vitro* [44, 45]. NCPs may regulate the size and orientation of the mineral deposits [3, 6]. Through chelation of calcium or enzymatic release of phosphorus from these proteins, they may serve as a reservoir for calcium or phosphate ions for mineral formation [3, 6]. These proteins are also supposed to act as linkers, attaching extrafibrillar mineral to the collagen fibrils, and mineralized collagen fibrils to each other. Bone sialoprotein (BSP), one of the most abundant NCPs in bone, was indeed described as bound to collagen type I [46]. Raif and Harmand [43] categorized these NCPs as either mineral-bound or organic-bound and further suggested that the mineral-collagen link is formed by a complex NCP network. Many matrix proteins are also thought to be involved in bone remodeling by participating in recruitment and attachment of cells to bone [38, 39].

Mechanisms involved in **mineral-collagen interfacial bonding** have been studied for a long time, most of the studies trying to identify factors that influence the adsorption of macromolecules on hydroxyapatite (Table 2) [47]. The interface between collagen and apatite crystals is still poorly understood. Interfacial bonding interactions between the mineral and organic phases of bone are due, in part, to the strong adsorption affinity of HA for organic material. This adsorption involves:

. electrostatic (coulombic) interactions between negatively charged organic domains (the anionic domains of proteins, that are the carboxyl groups) and the positively charged mineral surface (calcium) [47, 48]

. hydrogen-bonding networks [13, 47]

. and other types of interactions (van der Walls', hydrophobic bondings) [47, 48].

A number of cationic protein binding sites may be involved between the mineral and organic constituents of bone [48].

Forces involved in the interaction

A. *Coulombic attraction* between
- **a.** Positively charged Ca ions at the crystal surface and negatively charged carboxyl, sulfate, phosphate, or other groups on the macromolecule
- **b.** Negatively charged phosphate or OH ions at the crystal surface and positively charged amino or other groups on the macromolecule

B. *van der Waals' forces*
- **a.** Polar, e.g., charge-dipole interaction or charge-induced dipole interaction
- **b.** Nonpolar – dispersion forces

C. *Hydrogen bonding*, e.g., between HO-groups of carbohydrate and F or O atoms at crystal surface

1) Indirect interactions involved in stabilization of the adsorbed phase

A. Intermolecular attraction (loop interaction) involving van der Waals' forces and H bonding

B. Gain in entropy due to displacement of many "structured" water molecules from the apatite surface by a single polymer chain vs loss of entropy due to restriction in freedom of adsorbed molecule

2) Other factors affecting the adsorption process

A. Properties of the hydroxyapatite surface, e.g., net charge, nature of counterions, and surface configuration

B. Properties of the macromolecule, e.g., net charge, size, shape, flexibility, and solubility

Table 2: Factors possibly influencing the adsorption of macromolecules on hydroxyapatite [47].

1.1.2 Bone Remodeling

Both cortical and trabecular bone are constantly remodeled by a specific cycle of cellular activity, responsible of a dynamic process known as bone remodeling or turnover [49]. The basic metabolic unit (BMU) remodeling [50] consists of a group of all the cells (osteoblasts and osteoclasts) that participate in remodeling a certain area of bone through a sequence of cell activity consisting of activation, resorption and formation (Figure 10). The end result of this BMU remodeling cycle is called a bone structural unit (BSU) [51]. In cortical bone, it constitutes a Haversian system (or cortical osteon) and in trabecular bone, it is a wall or "packets" of bone.

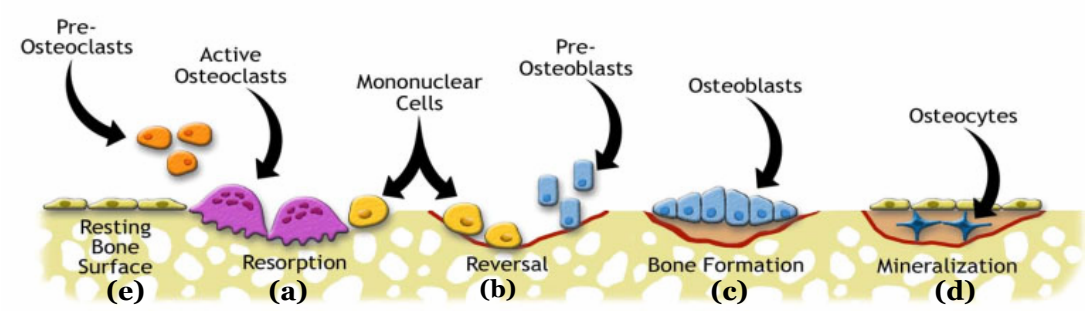

Figure 10: Bone Remodeling Cycle [52]

The first stage of remodeling is ***bone resorption*** (Figure 10a), lead by osteoclasts. Two mechanisms are thought to be involved in this resorption process: secretion of hydrogen ions (H^+) that dissolve the mineral lattice and action of proteases that degrade the organic matrix. Once the resorption pit is complete, osteoblasts are recruited. Resting

osteoblasts are activated to line the resorption pit during the ***reversal stage*** (Figure 10b) and then to begin ***bone formation*** (Figure 10c). Osteoblasts deposit osteoid matrix (unmineralized collagenous matrix) which will then undergo ***mineralization*** (Figure 10d). In human bone, osteoid experiences 65-70% of its final mineralization after 5-10 days [29]. After mineralization is completed, osteoblasts come back to a ***quiescent stage*** (Figure 10e). During this quiescent phase, secondary mineralization can occur, as described previously.

Cortical bone remodeling proceeds via cutting cones: the head of the cutting cone consists of osteoclasts that resorb bone, closely followed by a capillary loop and a population of osteoblasts that actively lay down osteoid to refill the resorption cavity [53] (Figure 11). By the end of the process, a new osteon has been formed.

The ratio of the mineral and organic phases of bone is maintained through this dynamic bone remodeling process. In general, trabecular bone material is much more active metabolically, is remodeled more often than cortical bone, and is therefore "younger" on average than cortical bone [3]. This different turnover rate observed in cortical and trabecular bone may be explained by the presence of larger trabecular surfaces undergoing remodeling as well as a larger number of active cells in trabecular bone. The lower turnover rate described in cortical bone may explain the lesser regional heterogeneity observed in this type of bone [3].

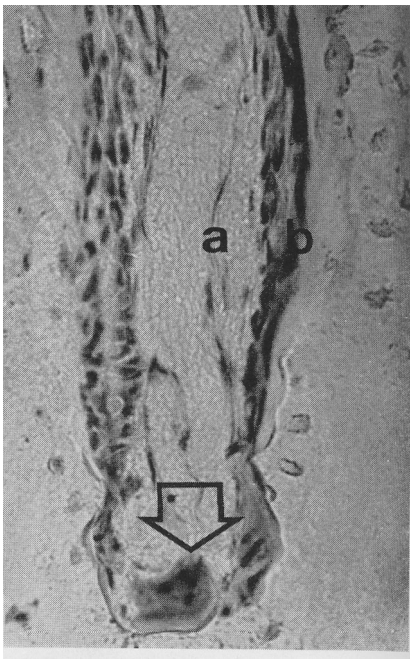

Figure 11: Longitudinal section at the extremity of a Havers canal [53].
Cortical bone remodeling proceeds via cutting cones. Cells are coming out of capillaries (a). Old bone is removed by osteoclasts (arrow), forming the cutting cone. Osteoblasts (b) begin to synthesize osteoid (filling cone).

1.1.3 Types of Bone

At the microscopic level, there are two types of bone, lamellar and woven bone. ***Lamellar bone*** is the most abundant bone structural type in many mammals, including humans [5]. A lamellar unit is composed of five sublayers. Each sublayer is an array of aligned mineralized collagen fibrils. The orientation of these arrays differ in each sublayer with respect to both collagen fibril axes and crystal layers, such that a complex rotated plywood-like structure is formed [4, 5]. Studies on lamellar bone mechanical properties have demonstrated a clear-cut anisotropy with respect to the axis direction of long bones, and have shown that most of the intrinsic mechanical properties were built into the lamellar structure [5]. Lamellar bone is present in two basic forms: as extended parallel arrays of lamellae (called ***circumferential lamellar bone***) or in the form of cylinders, which are referred to as ***osteons*** [54] (Figure 12). The osteons can fill spaces left during the rapid growth of parallel-fibered bone, to form a mixed bone type called "fibrolamellar bone". These are called ***primary osteons***. Alternatively osteons are produced secondarily after removal of earlier formed bone during the remodeling process. These secondary osteons are usually cylindrical and are known as ***Haversian systems*** [5]. De Ricqlès *et al* [55] have pointed out that only large animals tend to produce bones that are composed almost entirely of osteons, whereas in smaller animals the initially deposited primary lamellar bone is often left intact. But there is no well-defined cut between "large" and "small".

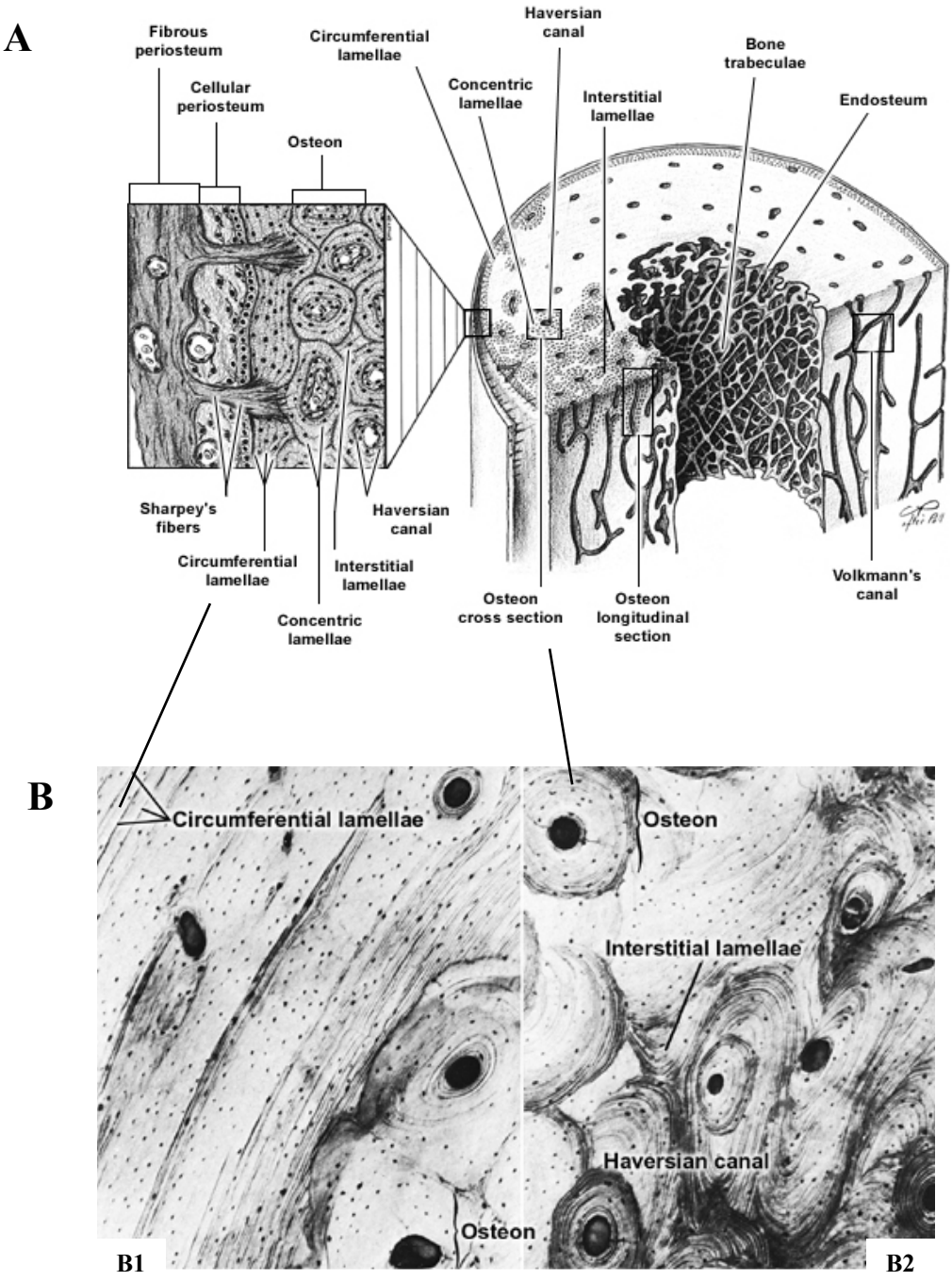

Figure 12: A-B. Lamellae can be organized as paralleled arrays (circumferential lamellar bone) or as cylinders, forming osteons [7].

B. Ground sections of dried cortical bone [7]: near the outer surface (B1); central portion (B2).

Another form of bone where the mineralized collagen fibers are less well registered and no pattern can be distinguished is called ***woven bone*** [3]. Woven bone is common in the skeletons of amphibian and reptiles, as well as in the skeleton of the mammalian embryo and in the metaphyseal region of growing bone. During development, embryonic woven bone is replaced by other bone types (Figure 13). Woven bone is also the first bone type to be formed after fracture or in other pathological circumstances in which rapid bone formation is a prime concern [4], such as tumors, osteogenesis imperfecta and pagetic bone [56].

Woven and lamellar bone are structurally organized into trabecular (or cancellous) bone and cortical (or compact) bone. The name of the **trabecular bone** derives from its architecture: it consists of trabeculae (or struts) that are osseous structures having a platelike (P) or rodlike (R) configuration, which interlace and anastomose to form a spongy structure (Figure 5). In trabecular bone, only about 20% of the volume is filled with bone material and the rest with bone marrow. The microarchitecture of trabecular bone is not random: the orientation and connections of the trabeculae have precise patterns which are believed to be related to the specific mechanical properties [57]. Trabecular bone is found inside cuboid bones such as vertebral bodies, and in the epiphysis and metaphysis of long bones.

Cortical bone is a complex of many adjacent osteons, surrounded by their interstitial and circumferential lamellae (Figure 12). It is found in the diaphysis of long bones and in the outer shell of cuboid bones.

Both types of bone demonstrate a very different porosity: the porosity of cortical bone varies from 5 to 30%, while the porosity of trabecular bone ranges from 30 to more than 90% [57].

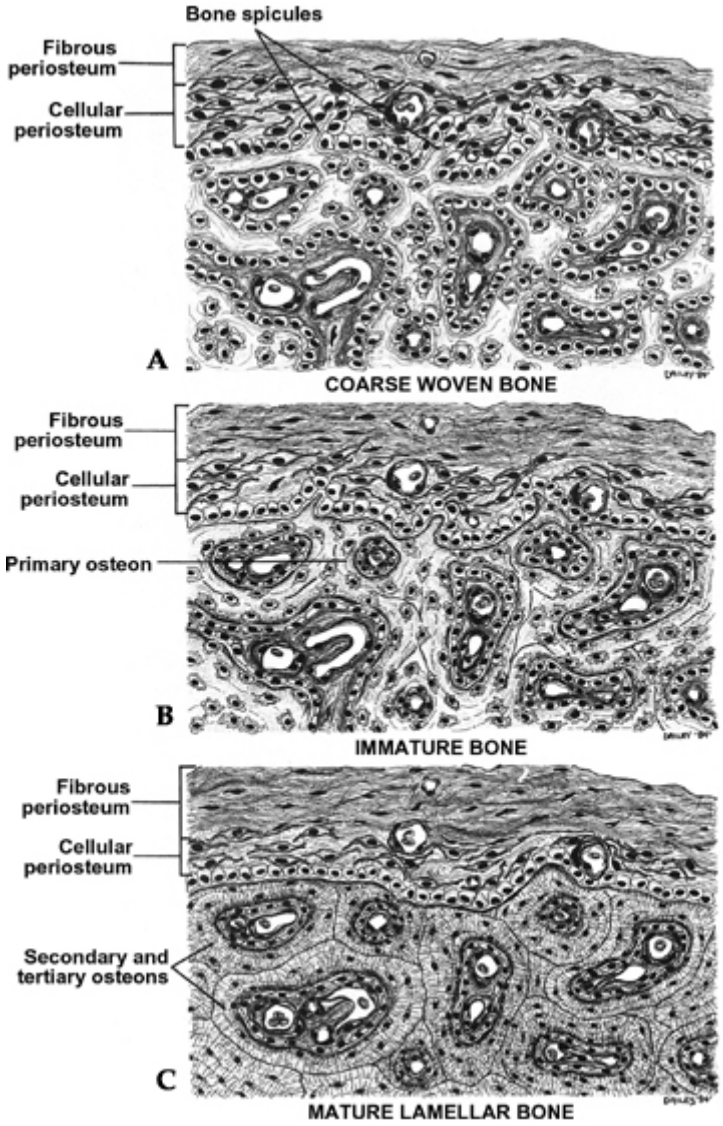

Figure 13: Woven bone is cellular and disorganized. In mammals, it is a transitory type of bone that will be replaced by a more mature type of bone [7].

1.2. Bone Quality

The expression "bone quality" is a vague term that is used in two ways in the literature [58]. In one usage, bone quality represents the sum of all characteristics of bone that influence the ability of bone to resist to fracture [59, 60]. In another usage, bone quality refers to the influence of factors that affect fracture but are not accounted for by bone mass or quantity [61, 62]. Nowadays, there is no current consensus regarding the definition of "bone quality" [59]. In the present work, we will use the first of these two definitions.

All of the hierarchical levels of bone complexity influence the mechanical behaviour of bone [3]. A number of characteristics of bone have been implicated as important aspects of bone quality [58, 59, 60, 61, 63] (Table 3). Mechanical parameters are directly affected by bone structural parameters, like cross-sectional geometry, and by the material properties of the bone tissue [64, 65, 66, 67, 68, 69, 70, 71, 72]. However, the relative importance of structural and intrinsic material properties is still unknown.

Scale (m)	**Bone characteristics**
$>10^{-3}$	• Whole bone morphology (size and shape) • Bone density spatial distribution
$10^{-6} – 10^{-3}$	• Microarchitecture • Porosity • Cortical shell thickness • Lacunar number/morphology • Remodeling cavity number, size, and distribution
$10^{-9} – 10^{-6}$	• Mineral and collagen distribution/alignment • Microdamage type, amount, and distribution
$<10^{-9}$	• Collagen structure and cross-linking • Mineral type and crystal alignment • Collagen-mineral interfaces

Table 3: Physical and chemical characteristics of bone that may influence biomechanical bone quality, categorized by physical scale [58].

Collagen matrix

It is generally accepted that collagen fibrils, mineral platelets and water are important for the mechanical properties of bone, whereas this role has only recently been suggested for the NCPs [73]. The contribution of the mineral phase to bone's mechanical properties has been studied extensively, while collagen's role has been less studied. It is believed that, in structurally normal bone, the mineral provides stiffness and strength, whereas collagen affords ductility and toughness [74, 75, 76, 77, 78]. The collagen intermolecular cross-linking provides the fibrillar matrices with various mechanical properties [79] such as tensile strength and viscoelasticity [21]. There is ample evidence in the literature that changes to the collagen molecule alone can alter bone quality and therefore fracture risk [77, 80]. However the influence of the specific interaction between mineral and collagen on bone mechanical properties is still poorly understood [78].

Hydroxyapatite crystals

The size, shape and ***arrangement*** of the apatite crystals in bone are major determinants in establishing its biomechanical properties. Mechanical models of bone show the importance of having oriented nanometer-sized mineral crystallites in a collagen matrix to the mechanical properties of bone [3]. This organization may contribute to the roughness of the mineralized fibrils, especially in tension along the fibrils [73]. In this nanoscale friction mechanism, the mineralized fibrils would be resistant to sliding relative to each other because of mineral plates on one mineralized fibril hitting mineral plates on another mineralized fibril.

At least two different aspects of the mineral component of bone affect its mechanical properties: the ***degree of mineralization*** of bone (mineral:collagen ratio) and the ***degree of crystallinity*** of the mineral (i.e., grain size and/or length scale of structural continuity

within a grain). Akkus *et al.* [35] showed that age-related changes in physicochemical properties of mineral crystals (increasing mineralization, increasing crystallinity and increasing type-B carbonate substitution) were significantly correlated with decreasing elastic deformation capacity in rat cortical bone.

Water

Wilson *et al.* [13] hypothesize a role for water in mediating mineral-organic matrix interactions. Water may help to stabilize the mineral structure (crystal-bound water that occupies vacancies in the imperfect carbonated apatite crystal lattice, may provide structural stability by forming hydrogen-bonding bridges between neighboring ions) and may have a role in coupling mineral crystallites to the surrounding bone structures (the water layer observed at the surface of bone mineral crystallites may mechanically couple the mineral and collagen). Water located at the surface of mineral crystallites may serve as a cushion against mechanical stress.

Noncollagenous organic proteins

Despite the fact that the NCPs make up only a few percent by mass of the bone material, more and more studies indicate that they must play an important role in bone mechanical properties and thus in bone quality [14, 15, 43, 46].

Organic-inorganic interface

Bone interfacial properties are more and more studied and are thought to play an important role in the behaviour of bone at the macroscopic scale. The importance of the organic-inorganic interface for the mechanical properties in bone has been emphasized in earlier works [48]. The exact nature of this interface between the main organic (collagen fibrils) and inorganic (HA) components of bone is still a matter of ongoing

debate. The calcified HA crystal surfaces are bridged with the negatively charged polyelectrolytes (i.e. NCPs), attached to the organic matrix (collagen type I) [39, 81]. Hydrogen-bonding networks and NCPs are thought to cooperatively stabilize this interface [13]. Fantner *et al.* [73] have described the presence of a non-fibrillar organic matrix which acts as a "glue" that holds the mineralized collagen fibrils together and could resist their separation. When a force is applied on the bone, the glue is stretched and energy is dissipated in the glue through rupturing of sacrificial bonds and the stretching of hidden length. Sacrificial bonds are additional, weak but reformable, bonds that break before the strong bonds that hold the structure together break. The energy required to break these weak sacrificial bonds increases the total energy needed to fracture the material, thereby increasing the toughness of the bone. The exact nature of the bonds and molecules involved in this mechanism is still unknown. Fantner *et al.* [73] hypothesized that these include calcium-mediated sacrificial bonds. These sacrificial bonds are thought to exist within collagen fibrils, between mineralized collagen fibrils and between collagen fibrils and mineral plates. Many noncollagenous bone matrix protein have negatively charged groups such as phosphate groups on phosphorylated amino acids at physiological pH that could be bound together into sacrificial bonds by multivalent positive ions and are thus natural candidates for this "protein-based glue" [82].

Microdamage and bone remodeling

The presence of microdamage and the efficacy of bone remodeling are also important factors that influence bone quality [59, 83]. Bone strength depends on the structural and material properties of bone, both of which are influenced by the rate of bone turnover. It is generally accepted that in "healthy" bone, there is a balance between accumulation of damage due to daily loading and its repair by activation of remodeling

[84]. Any changes in bone remodeling that alter this critical balance between damage accumulation and repair, may then modify bone mechanical properties [59].

Bone anisotropy

Bone material is not homogeneous and isotropic [6]. Both cortical and trabecular bone are anisotropic materials, meaning that their mechanical properties depend on the loading direction. This anisotropy explains the large local variation observed in material properties of bone. Variations in trabecular and cortical bone quality across skeletal regions have been described [58, 85, 86]. This may be explained by differences in bone microarchitecture (plate-type trabecular bone is much more mechanically efficient than rod-type trabecular bone) or variations in tissue material properties, which may be associated with degree of mineralization [65, 87] or quality of organic-inorganic interfacial bonding [88]. This heterogeneity has been proposed as playing an important role in bone mechanical integrity [89]. Burr [90] proposed that homogeneous materials were generally poor at resisting crack propagation and had reduced toughness. Therefore, discontinuities in the bone matrix mineralization might have an important influence on the crack initiation and propagation behaviour [21].

In conclusion, bone quality encompasses parameters that contribute to the mechanical integrity of the bone. The bone quality may be defined as the result of the structural and intrinsic material properties and also of the interaction between bone morphology and bone material properties. Indeed, functional interactions among bone traits have been shown to be critical for mechanical functionality [91, 92].

1.3. Bone and Genetics

Nowadays, it is well established that genetics can substantially influence bone in many aspects such as bone mass and bone mineral density [93, 94, 95, 96, 97, 98, 99], bone morphology [97, 99, 100, 101], bone material properties [91, 97, 99, 102, 103], bone mechanosensitivity [104, 105, 106], bone remodeling process [100, 107] and structure-function relationships (i.e., the correlation between whole bone mechanical properties and the underlying physical bone traits) [108]. The past few years have witnessed real advances in ultrahigh-throughput genotyping capabilities and cataloguing of millions of sequence variations across the human genome [109]. These advances have enabled well-powered genome-wide association studies and nowadays we can find in the literature more and more reports demonstrating clear links between genetic polymorphisms and some medically relevant traits as bone mineral density [110, 111], or some multifactorial diseases as osteoporosis [110, 112, 113]. Such discoveries have real clinical impacts, allowing to investigate new therapeutic protocol [114]. Furthermore individual genetic profiles, in addition to information on other risk factors, may one day enable targeting of specific therapeutic strategies to those at greatest risk of developing specific diseases such as osteoporosis [115]. Nevertheless it must be pointed out that genome-wide association studies do have limitations. For example, efficient methods to detect possible gene-gene or gene-environment interactions, which may have large effects on a phenotype [116], are still needed [115]. Environmental factors are known to have a potential influence on bone properties [94] and this action may also be influenced by the individual's genetic background [116].

1.4. Fluoride

1.4.1 Fluoride as a Trace Element

Fluoride (F^-) is a trace element that is ingested through food, water, and fluoride containing dental products. Many foods contain F^- [117], mainly marine products. Fluoride ingestion from food in humans over the age of 12 is estimated at 0.4 mg/day [118]. In some countries, municipal water is fluoridated to prevent dental decay. Fluoride concentration in fluoridated water is usually around 1 ppm (1 μg/ml) [119] and the average adult consumption of water is estimated around 1.5 l per day. Individuals, especially children, also ingest F^- from oral products, particularly toothpaste [120]. In North America, toothpaste is fluoridated at 1000-1100 ppm. If it is considered that 1 g of toothpaste is usually used per brushing and that adults ingest about 25% of it [121], it means that adults ingest 0.25-0.75 mg of F^- each day. Overall F^- consumption is estimated to range from 1.2 to 2.2 mg/day in adults [121]. Under most conditions, F^- is rapidly and extensively absorbed from the gastrointestinal tract. Fluoride removal from plasma occurs by calcified tissue uptake and urinary excretion. About 99% of the body burden of F^- is associated with calcified tissues, and most of it is not exchangeable [121]. The clearance of F^- from plasma by the skeleton is inversely related to the stage of skeletal development. Fluoride is known to accumulate only in the new bone deposited during the period of exposure [119]. It is not incorporated into mature, fully mineralized bone that is not subjected to any remodeling. The amount of F^- that is incorporated in bone may be influenced by various factors, like diseases affecting bone remodeling or renal function [121]. In osteoporosis, for example, as there is an

unbalance in bone remodeling in favour of bone resorption, F^- is less incorporated into bone. The accumulation of F^- into bone is thus positively associated with bone remodeling rate [122], and will be more important in bone demonstrating the highest turnover rate, like trabecular bone (in opposition with cortical bone) [123, 124]. Fluoride can be cleared from the skeleton by bone remodeling, but F^- clearance takes over four times longer than F^- uptake [122].

Many studies have described the occurrence of dental and skeletal fluorosis in case of chronic exposure to high F^- dose levels [125, 126]. It must be noted that the definition of the expression "fluorosis" is clinical: it is used when chronic exposure to excess fluoride induces toxic effects, predominantly dental [127] and skeletal [128]. Nowadays, fluorosis is still a serious public health problem in many part of the world [126, 129, 130, 131, 132, 133]. In some areas, drinking water contains high amounts of F^-, and dental as well as skeletal fluorosis is endemic. At the present time, the recommended intake of F^- in the diet is still a controversial problem [134, 135, 136].

1.4.2 Fluoride Action on Bone Cells

Osteoblasts

Numerous *in vivo* studies agree that F^- stimulates osteoblast proliferation and bone matrix synthesis in humans [137] and in animals [138, 139]. Some *in vitro* studies have demonstrated that high serum levels of F^- induced an increase in osteoblasts activity by acting directly on osteoblasts to increase their proliferation rate and their alkaline phosphatase activity [140, 141], while other *in vitro* studies did not show any evident direct effect of F^- on osteoblasts [142, 143, 144], suggesting that F^- could act

either directly on osteoprogenitor cells in bone marrow or indirectly through a cofactor absent from the culture system. However the osteoblasts appear to be flattened and only moderately active, rather than plump and highly secretory [119]. Therefore, it is believed that, despite its mitogenic effect on osteoblasts and/or promoter effect of differentiation of osteoblast precursors, F^- may be toxic at the individual cell level [139]. This toxic effect on osteoblasts is responsible of a reduced osteoblast activity, a prolonged formation period and a delay in the onset of the mineralization process [139]. *In vivo* and *in vitro* studies [145, 146] have shown that excessive F^- doses can induce a decrease in the content of type I collagen and in the degree of collagen cross-linking in rat bone. They suggest that F^- can affect the protein synthesis in osteoblasts by changing the expression of the related genes.

Osteoclasts

The effect of F^- on osteoclasts is less well understood. *In vitro* studies have shown that sodium fluoride decreased the number of resorption lacunae as well as the resorbed area per osteoclast [147]. The possible influence of F^- on osteoclastogenesis and osteoclasts could be mediated by an action of F^- on PTH secretion [148].

The net result of these effects of F^- on bone cells is increased bone formation [119, 139]. It is usually accepted that high F^- concentrations are necessary for F^- to affect bone cells. Turner *et al.* [149] describe drinking water F^- contents up to 4 ppm as a reasonable threshold below which the effect of F^- on bone cells is negligible.

1.4.3 Fluoride Action on Bone Mineralization and Hydroxyapatite Crystals

In vitro studies have well described the effects of F^- on bone crystallites. These studies show that F^- ions interact with apatite type structures (HA) in different ways depending on the concentration and the pH of the medium [150, 151]. At low concentrations (≤0.001 M), the exchange of F^- with hydroxyl ions of apatite generates a FHA (fluorohydroxyapatite) type material [150]. In high concentrations, part of the bone mineral dissolves and reprecipitates mostly as calcium fluoride (CaF_2) with some phosphate ions and fluorapatite/fluorahydroxyapatite type of material [152]. A major limitation to these studies is that we do not know whether or not the alteration in bone mineral that occurs *in vitro* matches that which occurs *in vivo*. It is generally accepted that *in vivo*, F^- ions substitute for the hydroxide ions of apatite crystals, making the apatite lattice more stable and less soluble [29, 153, 154] (Figure 14). This ionic exchange has been demonstrated to be incomplete (even in highly fluorotic bone, F^- replaces only about a third of the hydroxyl ions) [48, 155] and irreversible [156]. Theoretical and experimental results suggest that this substitution occurs superficially, eventually resulting in precipitation of CaF_2, which is then layered on the hydroxyapatite surface [48]. Given the stabilizing role of the organic-inorganic interface, the fluorination of the calcified interface may lead to the disordering of this highly complex network [157].

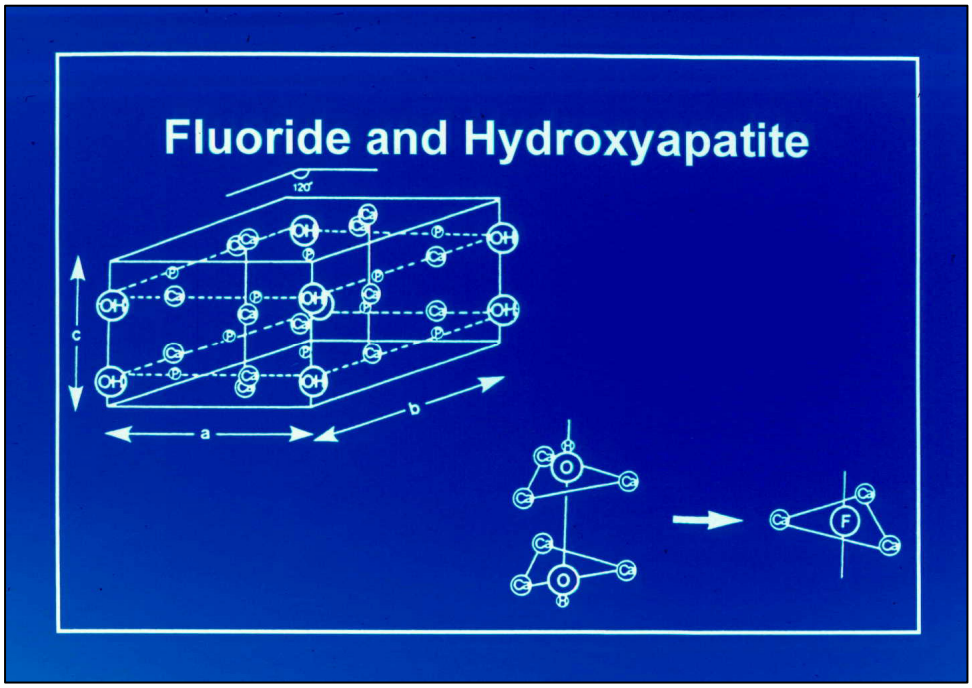

Figure 14: schematic showing the three-dimensional organization of atoms in a unit cell of hydroxyapatite (left) and diagram showing substitution of fluoride ions for hydroxyl ions [153].

Fluoride also influences the chemistry of the bone mineral, especially the trace elements in the lattice of the crystal and on its surface [29, 154]. The most important change is the drastic decrease in citrate ions, which seems to correlate with the decrease in surface area [153, 158]. Fluoride also influences bone crystal size, but this effect is more controversial. Though most authors showed no changes in crystal length [159, 160], some found an increase in crystal width [159, 161, 162, 163, 164] and others did not find any change [160].

It has been observed that F^- delays the initiation of mineralization of newly formed bone matrix and decreases the rate at which mineralization proceeds on

previously calcified matrices (mineralization lag time) [141, 155]. Effect of F^- on putative precursor phases in the formation of bone mineral apatite has been proposed, but this hypothesis is based on *in vitro* studies [161].

Detrimental effects of F^- on bone mechanical properties have recently been ascribed to a weakening of the organic-mineral interfacial bonding [48, 88, 165, 166]. However, the precise effect of F^- on bone ultrastructure is still a matter of ongoing discussion and no direct observation of the hypothesized organic-mineral interface failure exists to date.

In summary, by changing the structure of the apatite crystals (increased crystallinity, reduced crystal strain, reduced unit cell size, reduced solubility, reduced specific surface area), F^- can affect bone mineral deposition and dissolution. By making bone mineral less susceptible to acid dissolution, F^- may alter the kinetics of bone resorption. Fluoride may also have an effect on bone matrix by reducing the amount of proteins bound to bone mineral due to the reduction of the specific surface area resulting from an increased packing of bone crystals [153].

1.4.4 Fluoride Action on Bone Mechanical Properties

Consistent with clinical studies on osteoporosis treatment with sodium fluoride [167, 168, 169], it has been observed that the relationship between trabecular bone strength and apparent density is compromised by sodium fluoride treatment [170, 171]. Nowadays it is well demonstrated that the mechanical properties of rodent bones treated

in vitro or *in vivo*, are degraded in a dose-dependent fashion as F^- content increases [149, 172]. The effects of *in vitro* F^- exposure on bone mechanical properties have been investigated by some authors [172, 173]. Fluoride exposure has been shown to affect both torsional and bending properties of whole bones in mice [172]. Walsh *et al.* [48] demonstrated a reduction in compressive properties of bovine cortical bone after *in vitro* F^- ion treatment. *In vivo* studies have also reported negative effects of dietary sodium fluoride on bone mechanical properties [174]. Turner *et al.* [162] showed that high-dose F^- treatment decreased bone strength in rabbits' femoral necks and L5 vertebral bodies.

1.5. Mouse model

1.5.1 Utility

Animal models produced by means of artificial selection are used worldwide for studying different conditions in humans. Many studies use the mouse as a model organism because of their ease of breeding and reproductive capacity, their short life span and the relative ease of genetic manipulation coupled with the extensive genomic, anatomical and physiological synteny with humans [107, 175, 176].

Inbred mouse strain represents a genetically homogenous group that often differs quite remarkably from mice in another inbred strain. A strain is regarded as inbred when it has been mated brotherXsister for at least twenty consecutive generations and can be traced to a single ancestral breeding pair in the 20^{th} or a subsequent generation [177]. At 20 generations, on average at least 98.6% of the loci in each mouse are homozygous. Many strains have been bred for more than 150 generations and are essentially homozygous at all loci. Due to their isogenicity, inbred mouse strains demonstrate low biological variability within each strain [177]. Isogenicity within an inbred strain and the genetic heterogeneity between inbred strains allow to study the role of the genetic background in different phenotypes.

Over 450 inbred strains of mice have been described, providing a wealth of different genotypes and phenotypes [177, 178, 179]. As two related strains may show phenotypic and genotypic similarity, knowledge of inbred strains genealogies is critical to the design of experiments that require unrelated parental strains [180, 181, 182]. Many inbred strains are bred for specific phenotypes. The crossing of phenotypically different inbred strains allows the mapping of quantitative or qualitative trait loci. Analysis of a

complex trait involves choosing phenotypically and also genotypically distinct parental strains because genetic mapping depends on the polymorphic differences between parents. Nowadays more than 2,000 quantitative trait loci (QTLs) have been identified in crosses between inbred strains of mice [183]. Strictly speaking, a QTL is any locus that contributes to a phenotype that is measured quantitatively. Different strategies are available to identify genes that underlie QTLs [183]. Inbred strains of mice may thus prove useful for identification of the QTLs and finally genes that are associated with the studied phenotype.

Nowadays comparative map of genes that have been mapped in both mouse and human is available. An important application of comparative gene mapping is the possibility to predict the location of disease loci in one species given their location in another species [175].

Another advantage to use a mouse model system is the continuous eruption of the incisors. The continual growth of mandibular and maxillary incisors permits the effects of F^- on new enamel formation to be monitored [184, 185], allowing to correlate the effect of F^- on teeth with the F^- effect on bone.

1.5.2 Mouse compared to Human

Mice is a convenient source of well-mineralized bones. Nevertheless their bones have some characteristics that differ from human bones. Mouse bone does not undergo internal remodeling to form Haversian systems [2, 186] and therefore does not display active intracortical remodeling. Despite these differences, it is believed that the mechanism of F^- action on the bones of mice, rats and humans is the same and that

changes induced by F^- treatment in mice and rats bones can be directly applicable to human bones [149].

Another disadvantage to using the mouse model is the usually small size of their skeleton. Technical difficulties may result from mouse bone small size in assessing bone quality.

In general, most mouse strains present the same skeletal development as in human, with the same different phases including early rapid growth, attainment of peak bone mass, and age-related bone loss phases. Although mouse life span varies among different strains, the average lifespan is 16-20 months [187]. Depending on the strain, trabecular and cortical bone mass rapidly increases with age and peak bone density is reached at approximately 4 months of age [95].

While animal models are useful in providing information about F^- action on bone properties, there are two major differences that must be considered before extrapolating the results to humans. First, the rate of F^- incorporation is different in rodents and in humans. In rats, the rate of F^- incorporation is an order of magnitude smaller than in humans, suggesting that delivery of water fluoridated at 1 ppm to humans would be equivalent to 10 ppm in rats [149]. This is partially accounted for by differences in intestinal absorption [188]. Regarding mice, it has been shown that for the same dose of F^- in the drinking water, mice accumulate 6 times as much F^- in their femora than rats [153]. Secondly, animal studies typically examine the effect of F^- after short times treatments, rather than after long exposures to low doses, which is the situation mostly encountered in humans.

Nevertheless mice are a useful experimental model to study the influence of the genetic background on bone quality. As already noted, mice display known homologies with human genome. Indeed mice share 60-70% genetic similarity with humans [107]. Because of the close correspondence among mammalian genomes, identification of the

genes underlying bone strength in mammals such as the mouse is likely to be of major assistance in human studies.

1.5.3 Strains of Mice used in the Present Study

The role of genetics/genetic background in F^- responses has recently been demonstrated in dental fluorosis [189, 190]. Everett *et al.* [189] studied 12 inbred strains of mice to test the hypothesis that genotype can influence susceptibility or resistance to developing dental/enamel fluorosis. Variations in the onset and severity of dental fluorosis among strains were observed even though the strains demonstrated the same fluoride concentration in mineralized tissues (femora). Some strains developed rapid and severe dental fluorosis, whereas others were minimally affected, with minor dental fluorosis. These various strains were grouped into resistant, intermediate and susceptible strains. In this pilot study [189], the inbred strains of mice were selected upon their degree of unrelatedness from each other. The selection was also based upon availability, cost and strain usage in other studies. As we wanted to investigate the role of genetic background in the effects of F^- on the skeleton in these mice, we selected the strains that differed the greatest in their dental fluorosis. We chose to study the A/J and 129P3/J strains because they demonstrated very different genetic susceptibility to developing dental fluorosis, with A/J being a "susceptible" strain and 129P3/J being a "resistant" strain. The SWR/J strain had an intermediate phenotype.

CHAPTER 2: HYPOTHESES AND OBJECTIVES

2.1. Introduction

Fluoride is essential in the diet and is thought to be required for normal dental and skeletal growth [191]. The recommended allowance of F^- is 1 to 4 mg per day. Two clinical observations – the important increase in trabecular bone density of the axial skeleton in skeletal fluorosis and the low prevalence of osteoporosis in areas with moderately high fluoride concentrations in drinking water – have initiated the use of sodium fluoride (NaF) for the treatment of osteoporosis with vertebral fractures [192] in many European countries [137, 193, 194]. In the past and for more than 30 years, sodium fluoride has been a widely used therapeutic agent for established osteoporosis because of its anabolic effect on trabecular bone mass. Clinical and experimental studies have, however, indicated a detrimental effect of F^- on bone strength [195, 196]. Although F^- effects on bone mechanical properties have been studied for a long time [197], the exact mechanism of action of F^- is still matter of controversies. Most of the studies investigate the effect of *in vitro* F^- treatment on bone [48, 88, 157, 173]. But *in vitro* models may not represent the real situation occurring in vivo. There are some limitations to the *in vitro* models of F^- action on bone. The alterations in bone mineral that were described in *in vitro* studies may not match the *in vivo* bone crystallites alterations. In addition, the spatial distribution of F^- in bone is unknown and it has been shown that F^- does not accumulate uniformly in bone [198]. Furthermore, little is known regarding the influence of an individual's genetic background on the responses of bone to F^-. Some fluoride therapy studies have demonstrated that F^- can have a positive effect

on axial bone density but approximately one third of patients are non-responders [199, 200, 201]. This "resistance" to fluoride therapy observed in some patients, was also described by Riggs [202] who proposed to consider a special subgroup of osteoporosis which was characterized by failure of osteoblasts to respond to fluoride treatment. Although F^- concentration is an important parameter that can influence its toxicity [203], it is more likely that the dose level is not the only factor. Several studies [131, 204, 205, 206, 207] have demonstrated a difference in the prevalence of dental and/or skeletal fluorosis depending on the sex, age, and the origin of the population studied, despite the fact that all individuals received the same F^- dose. Choubisa *et al.* [131] have studied the osteo-dental fluorosis in children and adults from ten villages in Rajasthan. The prevalence of skeletal fluorosis was relatively higher in males and increased with higher F^- level and age. Yoder *et al.* [207] have studied the F^- water concentration in 3 villages localized at different altitudes in Tanzania and have examined 284 children who were lifetime residents in these villages. In one village, the severity of teeth disturbance was not consistent with the low F^- concentration in drinking water and was more severe than would be expected from the subjects' normal urinary F^- values. Some data [206] suggest that dental fluorosis is more prevalent among African-American people than among other ethnic groups in the same community even though they receive the same F^- concentration. Russel [204] surveyed 822 students for dental fluorosis in the seventeenth year of fluoridation in Grand Rapids, Michigan. He found that dental fluorosis was twice as prevalent in the African-American group than in the white children group in regions where the F^- concentration in drinking water was the same. In the study of Butler *et al* [205], a higher dental fluorosis prevalence was also found in African-American children, compared with Hispanic and non-Hispanic white children, in Texas. All these observations suggest that genetic determinants may influence individual's susceptibility or resistance to fluoride effects.

Presently only few studies explore an underlying genetic basis for F^- effects [208, 209], and to our knowledge, an animal study about the influence of the genetic background in skeletal fluorosis has never been published.

2.2. Hypotheses and objectives

The primary goals of the present study were to study the effect of increasing fluoride doses on bone properties and to test the hypothesis that genotype can influence susceptibility or resistance to develop bone fluorosis.

Specifically, we hypothesize that

1. All mice receiving the same dose of F^- will accumulate similar amounts of F^- in their long bones and vertebral bodies regardless of whether they exhibit mild or severe bone fluorosis.
2. The bone mineral density and the bone mineral contents of the lumbar spine and the femur will be more influenced by F^- treatment in the susceptible strain than in the other strains, for the same F^- concentration.
3. The mechanical properties of the trabecular and cortical bone will be altered by the F^- with the greatest effect in the susceptible strain.
4. The trabecular bone microarchitecture and histology will be modified by F^-, with the greatest modifications in the susceptible strain.
5. The accumulation of F^- in bone will influence the mineralization and the microhardness of the bone.

<u>Hypothesis 1:</u> The fluoride concentration in bone will be the same for every mouse in a same treatment group, whatever the strain.

Background: Bai X *et al.* [210] have recognized that the concentration of F^- in bones and teeth must be taken as key indexes. In their study, Everett *et al.* [189] have demonstrated that there was significant correlation between F^- concentration in water and F^- concentration in femur but that the mice treated with the same F^- dose accumulated similar amounts of F^- in their femora whatever the strain considered.

Objective: To demonstrate that every mouse in a same F^- treatment group will accumulate the same amount of F^- in their bones, whatever the strain, so that any difference that could be discovered between these strains cannot be explained by different concentrations of F^- in bone.

Experimental design: The F^- concentration in the left femur and vertebral bodies will be determined using Instrumental Neutron Activation Analysis (INAA). In INAA, each sample is bombarded with thermal neutrons that produce short-lived radioisotopes from the sample elements. These radioisotopes decay with specific half-lives, emitting gamma rays of characteristic energies. The relative amounts of gamma rays detected are proportional to the concentrations of elements in the sample [211].

<u>Hypothesis 2:</u> The bone density will be more affected in the susceptible strain.

Background: Some studies [162, 212] have demonstrated that F^- increases bone mass. None of these studies have examined the influence of the genetic background on this bone mass modification.

Objectives: The first objective is to explore if the genetic background can influence the previously reported effect of F^- on Bone Mineral Density (BMD). The second objective is to look for an eventual correlation between BMD changes and bone mechanical properties.

Experimental design: DXA (Dual energy X-ray Absorptiometry) scan of the lumbar vertebral body and femur will be performed using PIXIMUS, a dedicated small animal DXA machine. Bone Mineral Content (BMC), bone area and Bone Mineral Density (BMD) will be determined for vertebrae and femora [213].

<u>Hypothesis 3:</u> The mechanical properties of trabecular and cortical bone will be mainly altered in the susceptible strain.

Background: A few studies [162, 212] have demonstrated that the bone mass increase induced by F^- treatment was not associated with an improvement of bone mechanical properties, but rather with decreased bone strength. This is in contradiction with other studies that showed no change or even improvement in bone mechanical properties with F^- treatment [193, 214].

Objectives: The first objective is to study the effect of increasing F^- doses on bone mechanical properties. The second objective is to determine if genetics can influence the bone mechanical properties changes that could be discovered.

Experimental design: Every mechanical testing will be performed using an Instron materials testing machine. The right femur (cortical bone) will be tested in three-point bending. Femoral ends from the three-point bending experiments will be tested for femoral neck fracture. The head-neck structure will be tested to failure by loading the head parallel to the shaft. The most distal lumbar vertebral body (trabecular bone) will

be tested under compression. From the load-displacement curves recorded, the ultimate load and stiffness will be determined.

<u>Hypothesis 4:</u> Fluoride will induce bone histological and connectivity changes that will be more severe in the susceptible strain.

Background: Some authors [155, 215] have performed histomorphometric analysis of undecalcified section in transiliac biopsy cores in patients suffering from skeletal fluorosis due to chronic exposure to fluoride. These studies found disturbance in the histomorphometric parameters, mainly with increased osteoid parameters (trabecular osteoid volume and perimeter, width of osteoid seams).

Objective: To determine if bone histomorphometric changes induced by F^- treatment can be influenced by genetic background.

Experimental design: Bone formation will be evaluated by static histomorphometry of a distal thoracic vertebral body. The percentage of total bone, mineralized bone and osteoid as well as the standard static histomorphometric parameters will be determined using a Leitz Bioquant morphometry system. Every histomorphometric data will be analysed and calculated according to the American Society for Bone and Mineral Research (ASBMR) histomorphometric nomenclature committee [216].

Bone microarchitecture will be quantified with microcomputed tomography and strut analysis of thoracic vertebral bodies.

<u>Hypothesis 5:</u> The accumulation of fluoride in bone will influence the mineralization and the material properties.

Background: Some studies [153, 160, 162, 164, 217, 218] have shown that there is an incorporation of F^- in the bone crystalline network and that the mineral crystal thickness is altered in fluoride-treated bones. While a lack of effect of F^- on crystal length is generally accepted, controversial effects of F^- on crystal width have been described [159, 160]. Regarding bone material properties, some authors demonstrated a decrease in bone microhardness [219], while others found an increase of the microhardness of both cortical and trabecular bone [164]. The exact effect of F^- on bone mineral crystals is still a matter of debate.

Objectives: To describe the effects of increasing F^- doses on bone mineral crystals and bone material properties, and to determine if any observed effect can be influenced by genetics.

Experimental design: Bone mineralization will be quantified with backscattered electron (BSE) imaging and powder X-ray diffraction.

Powder X-ray diffraction analysis will be performed on the left femur (after INAA) and will allow to calculate crystal size (length and width).

Mineralization distribution of the thoracic vertebral bodies and the distal end of femora will be evaluated using Backscattered Electron Imaging (BSE). The methodology has been described previously [220]. The image formed by the backscattered electrons exhibits a range of brightness that is related to the degree of mineralization.

Microhardness measurements will be taken from the thoracic vertebral bodies (cortical and trabecular bones) and the cortex of the distal femur. The hardness of bone is linked to the degree of mineralization and is proportional to bone stiffness.

The results are presented in two publications. The first publication explores the first three hypotheses, and studies the bone F^- concentration, the bone mineral densities and the bone mechanical properties. The second publication studies the structural and material properties of the bone.

CHAPTER 3: PUBLICATIONS

Mousny M, Banse X, Wise L, Everett ET, Hancock R, Vieth R, Devogelaer JP, Grynpas MD.

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The genetic influence on bone susceptibility to fluoride

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Abstract

Introduction: The influence of genetic background on bone architecture and mechanical properties is well established. Nevertheless, to date, only few animal studies explore an underlying genetic basis for extrinsic factors effect such as fluoride effect on bone metabolism.
Materials and methods: This study assessed the effect of increasing fluoride doses (0 ppm, 25 ppm, 50 ppm, 100 ppm) on the bone properties in 3 inbred mouse strains that demonstrate different susceptibilities to developing enamel fluorosis (A/J a "susceptible" strain, 129P3/J a "resistant" strain and SWR/J an "intermediate" strain). Fluoride concentrations were determined in femora and vertebral bodies. Bone mineral density was evaluating through DEXA. Finally, three-point bend testing of femora, compression testing of vertebral bodies and femoral neck-fracture testing were performed to evaluate mechanical properties.
Results: Concordant with increasing fluoride dose were significant increases of fluoride concentration in femora and vertebral bodies from all 3 strains. Fluoride treatment had little effect on the bone mineral densities (BMD) in the 3 strains. Mechanical testing showed significant alterations in "bone quality" in the A/J strain, whereas moderate alterations in "bone quality" in the SWR/J strain and no effects in the 129P3/J strain were observed.
Conclusion: The results suggest that genetic factors may contribute to the variation in bone response to fluoride exposure and that fluoride might affect bone properties without altering BMD.

Keywords: Fluoride; Bone; Genetic susceptibility/resistance; Inbred mouse strains; Bone mineral density

Introduction

Fluoride is an essential trace element whose action on bone has been extensively studied. Fluoride behaves as a cumulative element that can alter accretion and resorption of bone tissues as well as affect homeostasis of bone mineral metabolism [1–6]. At lower doses, fluoride can lead to an increase in bone mass and has been investigated as a therapeutic agent in adults for the treatment of postmenopausal osteoporosis [7–10].

Some fluoride therapy studies have demonstrated that fluoride can have a positive effect on axial bone density but approximately one third of patients are non-responders [11]. Although fluoride concentration is an important parameter that can influence its toxicity [12], it seems that the dose level is not the only factor. Several studies [13–17] have demonstrated a difference in the prevalence of dental or skeletal fluorosis depending on the sex, age and the origin of the population studied, despite the fact that all individuals received the same

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fluoride concentration. These findings suggest that genetic determinants may influence an individual's susceptibility or resistance to fluoride effects.

Everett et al. [18] studied 12 inbred strains of mice to test the hypothesis that genotype can influence susceptibility or resistance to developing dental/enamel fluorosis. Variations in the onset and severity of dental fluorosis among strains were observed, even though the strains demonstrated the same fluoride concentration in mineralized tissues. Some strains developed rapid and severe dental fluorosis, whereas others were minimally affected, with minor dental fluorosis. These various strains were grouped into resistant, intermediate and sensitive strains. Beyond this genetic study of dental fluorosis, few studies have explored the genetic basis for fluoride resistance [19,20]. To our knowledge, investigation into the role of genetic background in the effects of fluoride on the skeleton in an animal model has not been done.

The purpose of this study was to assess the effect of increasing fluoride doses on bone properties in inbred mouse strains in order to study the influence of genetic background.

Methods

Animal husbandry

Mice

Weaning (3-week-old) male mice from 129P3/J, A/J and SWR/J inbred strains were obtained from The Jackson Laboratory (Bar Harbor, ME). The strains were selected because of differences in susceptibility to developing dental fluorosis (A/J a "susceptible" strain, 129P3/J a "resistant" strain and SWR/J an "intermediate" strain) [18]. This study was fully approved by the Indiana University School of Dentistry IACUC, and all mice were housed within the Indiana University School of Dentistry Bioresearch Facility, a fully AAALAC accredited unit. Mice were contained in boxed caging and allowed food and water *ad libitum*. Mice were fed a laboratory rodent diet #5001 (PMI® Nutrition International, Richmond, IN) that contained by dry weight 0.95% calcium, 0.67% phosphorus, 4.5 IU/g vitamin D3 and 18 ppm fluoride.

Fluoride treatment

Four levels of fluoride as NaF (0, 25, 50 and 100 ppm F ion) were delivered in the drinking water from 3 weeks of age for a period of 42 days. Deionized and fluoridated water were periodically analyzed by ion-specific electrode (ISE) for fluoride concentration. Each batch of prepared 25 ppm, 50 ppm and 100 ppm water for the study was used only if the fluoride was within 4% of the target concentration. In the 3 strains, each treatment group consisted of 6 mice, except in groups A/J 25 ppm, A/J 50 ppm and 129P3/J 100 ppm which consisted of 7 mice. At the end of treatment, the mice were humanely euthanized. Selected bones were harvested for analysis.

Design of experiment

Preparation of bones

The lumbar vertebral bodies (VB) and femora (F) were dissected and kept moist with saline solution during the preparation and testing procedures. They were stored at −20°C. At the time of testing, the bones were thawed at room temperature.

Bone fluoride

Fluoride concentrations ([F], ppm) and the calcium percentages (% Ca, %) were determined in the left femur and the unused lumbar vertebral bodies using INAA (Instrumental Neutron Activation Analysis), as described by Mernagh et al. [21].

Bone density

The right femur and 4th lumbar vertebrae of each mouse were evaluated using the dual-energy X-ray absorptiometry PIXImus™ Mouse Densitometer (GE Medical Systems, Madison, WI USA) using software version 2.0. The instrument was calibrated daily using the manufacturer's phantom. Bones were individually positioned precisely at the center of the X-ray cone beam and scanned in air on the Plexiglas platform provided. Bone mineral content (g), bone area (cm^2) and bone mineral density (BMD; g/cm^2) were determined.

Mechanical testing and gross measurements

Mechanical tests were performed using a 100 N load cell on an Instron 4465 servohydraulic testing machine (Instron Corp., Canton, MA). The vertebrae were tested to evaluate the lattice network of trabecular bone, while femora were tested to evaluate cortical bone.

Three-point bending tests were performed on the right femur (Fig. 1A). A digital caliper (Mitutoyo, Hiroshima, Japan) accurate to 0.01 mm was used to measure femoral length from the greater trochanter to the femoral condyles. Each femur was positioned, anterior surface down, on two support bases separated by a constant 6 mm gauge length. A load was vertically applied to the midshaft of the femur at a rate of 1.0 mm/min until failure occurred. A load–time curve was generated using Labview Acquisition Software (LabVIEW 5.0, National Instruments, Texas, USA).

The average femoral cortical thickness at medial area, t_{avg}, was calculated as mean of medial, lateral, anterior and posterior cortex thickness at the fracture level after a 3-point bending test. External diameters in anteroposterior (D_1) and mediolateral direction (D_2) at midpoint were measured with the same digital caliper accurate to 0.01 mm. The internal diameters in anteroposterior direction (A_1) and mediolateral direction (A_2) were calculated following the formula "$A_{1-2}=D_{1-2}-2\times t_{avg}$". The cross-sectional area of the femur at midpoint was approximated following the formula "$\pi \times \frac{(D_1 \times D_2 - A_1 \times A_2)}{4}$".

Femoral neck-fracture tests were performed on the proximal section of the right femur following three-point bending (Fig. 1B). The distal part of this proximal section was embedded in a jig with PMMA and allowed to harden. The head–neck structure was tested to failure by loading the head parallel to the shaft at a rate of 0.5 mm/min. Load–time curves were generated.

For the vertebral compression test, unilateral compression was performed on the 4th lumbar vertebrae (Fig. 1C). Each vertebra was trimmed so that only the vertebral body remained. Images were taken of each vertebral body, using a photomicroscope at ×40 magnification. The cross-sectional area and the height of the vertebral body were then measured using ImageJ software (ImageJ 1.32j, 2004, NIH Image, USA). The distal end of the vertebral body was embedded in a shallow plate with PMMA so that the body was in an upright position. The load was applied to the top of the vertebra at a strain rate of 0.5 mm/min.

For all 3 testing modes, we generated load–deformation curves. From these curves, the ultimate load (N) and stiffness (N/mm) were determined. The stiffness was calculated as the slope of the linear portion of the curve. In the vertebral compression test, the 'failure point' was defined as the point where there was a 10% drop in the measured load.

Statistical analysis

Statistical analysis was performed on all parameters with SPSS (version 12.0) (SPSS Inc., Chicago IL) software. One-way analysis of variance (ANOVA, general linear model) was used to compare the results between the different treatment groups in a same strain as well as between the different strains at the same fluoride dose treatment to assess the influence of fluoride dose level and to study the genetic influence on fluoride effect. Post hoc multiple comparisons were performed using Dunnett t test. The Dunnett t test treats the 0 ppm treatment group as a control group and compares all other groups against it. A p value of <0.05 was required to consider the difference as significant. A confidence level of 90% (p value of <0.1) indicated a statistical trend. Data are presented as means ± SD.

Results

Fluoride concentration

Each strain demonstrated a dose-dependent increase of [F] in femora and vertebral bodies (Table 1). In each strain, fluoride dose had no significant effect on % Ca in either bone examined. At 100 ppm treatment, all 3 strains had comparable [F] in femora and VB.

Geometrical properties

Regarding the geometrical parameters of the femur (cortical thickness, length and cross-sectional area) and the vertebral body (averaged height, averaged cross-sectional area), there were no significant differences when we compared the F treatment groups within each strain.

Bone mineral density

In each strain, increasing fluoride dose did not influence BMD (Table 1).

Three-point bending test of the femora

Seventy-five three-point bending tests were successfully performed. The fluoride treatment only affected the mechanical properties in the A/J strain (Table 2). In this strain, there were significant decreases in ultimate load and stiffness with increasing fluoride dose treatment (Table 2). The ultimate load at 0 ppm treatment was significantly higher than the ultimate load at 50 ppm ($p=0.004$) and at 100 ppm ($p<0.001$) treatment. There was a significant difference between the stiffness at 0 ppm treatment and the stiffness at 100 ppm ($p=0.020$).

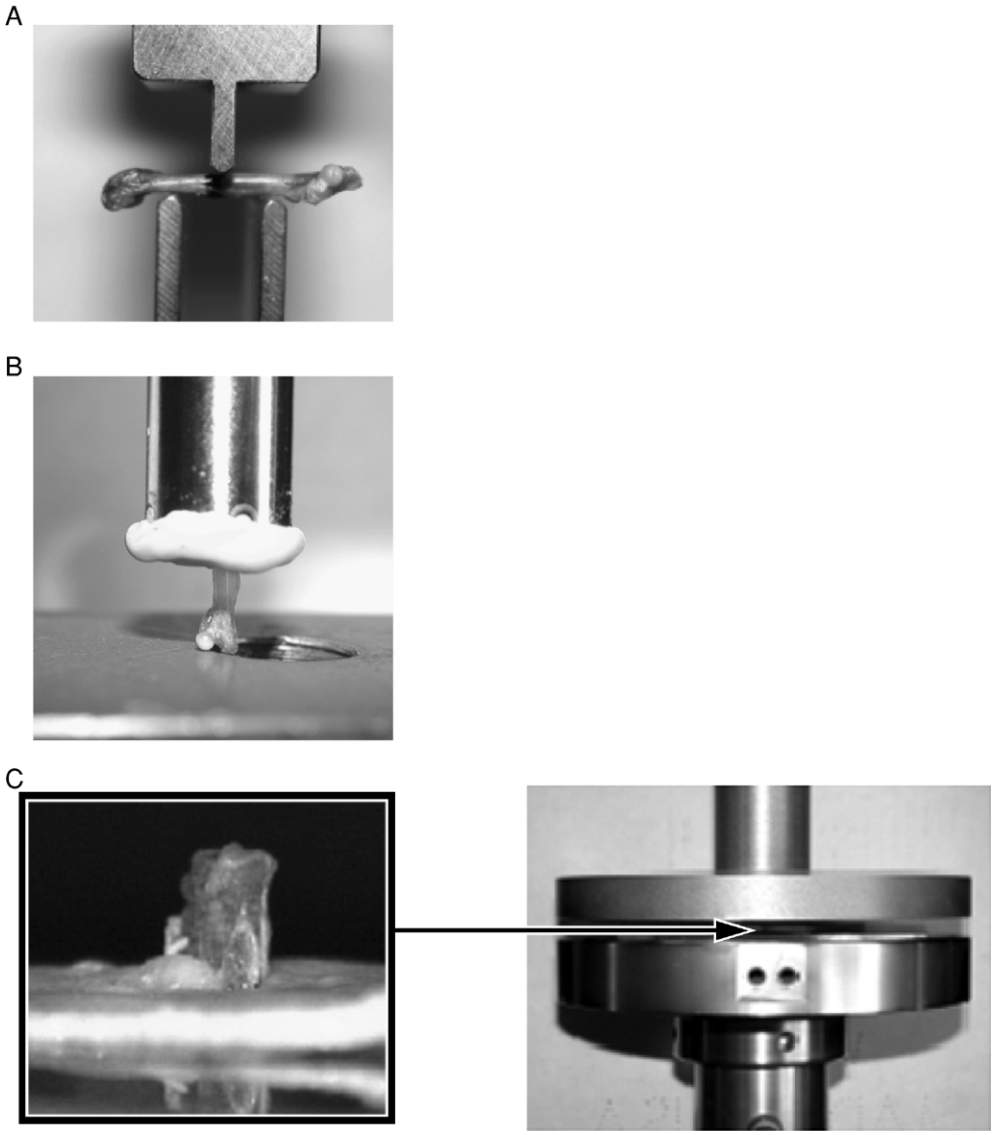

Fig. 1. Mechanical testing. (A) Femur three-point bending test. (B) Femoral neck-fracture test. (C) Vertebral body compression test.

Table 1
Fluoride (ppm), calcium (%) and BMD (g/cm^2) determinations in femora and vertebral bodies (VB) in each treatment group

	Fluoride treatment (ppm)	Strain A/J	Strain 129P3/J	Strain SWR/J
VB [F] (ppm)	0	90.8 ± 15.9	116.1 ± 14.3	97.4 ± 14.3
	25	416.7 ± 90.2***	512.9 ± 104.7***	616.6 ± 127.6***
	50	710.8 ± 133.4***	787.6 ± 82.9***	1097.3 ± 134.9***
	100	1472.2 ± 211.6***	1564 ± 265.6***	1712.4 ± 283.2***
VB % Ca (%)	0	7.91 ± 1.40	8.24 ± 1.07	7.92 ± 1.49
	25	8.26 ± 1.45	8.36 ± 1.68	9.15 ± 1.24
	50	7.12 ± 1.36	8.95 ± 0.98	9.14 ± 1.24
	100	7.40 ± 1.28	8.67 ± 1.41	8.71 ± 1.19
Femora [F] (ppm)	0	119.4 ± 20.9	176.6 ± 7.3	123 ± 17.2
	25	582.2 ± 87.1**	750.1 ± 41.9***	892.3 ± 121.2***
	50	1197.8 ± 171.6***	1116.2 ± 114.2***	1582.6 ± 264***
	100	2521.4 ± 334***	2435.9 ± 292.5***	2451.5 ± 346.7***
Femora % Ca (%)	0	15.81 ± 1.15	16.85 ± 0.95	15.77 ± 0.91
	25	16.91 ± 1.02	17.70 ± 0.95	15.89 ± 1.01
	50	15.62 ± 0.94	17.71 ± 0.68	16.46 ± 1.42
	100	15.77 ± 0.99	17.86 ± 1.76	16.02 ± 1.26
Femora BMD (g/cm^2)	0	0.050 ± 0.002	0.061 ± 0.002	0.050 ± 0.003
	25	0.050 ± 0.003	0.060 ± 0.004	0.051 ± 0.001
	50	0.048 ± 0.002	0.061 ± 0.003	0.052 ± 0.001
	100	0.048 ± 0.002	0.061 ± 0.002	0.047 ± 0.003
VB BMD (g/cm^2)	0	0.028 ± 0.003	0.035 ± 0.011	0.032 ± 0.003
	25	0.028 ± 0.002	0.035 ± 0.002	0.034 ± 0.002
	50	0.027 ± 0.003	0.034 ± 0.002	0.032 ± 0.001
	100	0.026 ± 0.002	0.035 ± 0.003	0.032 ± 0.002

Data are presented as means ± SD. A p value <0.001 is noted as ***. A $0.001<p<0.05$ is noted as ** and a $0.05<p<0.1$ is noted as *. At 0 ppm, 129P3/J exhibited the highest [F]. At 25 ppm and 50 ppm, SWR/J accumulated more fluoride than A/J and 129P3/J. At 100 ppm treatment, all 3 strains had comparable [F] in femora and VB.

Table 2
Mechanical properties of femora and vertebral bodies

	Fluoride treatment (ppm)	Strain A/J	Strain 129P3/J	Strain SWR/J
Femora ultimate load (N)	0	18.28 ± 0.86	25.07 ± 5.24	15.03 ± 1.80
	25	18.46 ± 1.21	25.04 ± 4.28	15.67 ± 0.93
	50	15.89 ± 1.49**	22.79 ± 4.07	15.44 ± 1.24
	100	12.85 ± 0.94***	22.19 ± 3.16	13.57 ± 1.93
Femora stiffness (N/mm)	0	128.93 ± 13.21	172.22 ± 31.04	105.69 ± 9.68
	25	136.99 ± 10.42	165.61 ± 15.33	110.36 ± 13.47
	50	118.50 ± 11.59	159.34 ± 31.55	101.91 ± 14.91
	100	108.45 ± 13.07**	153.98 ± 13.99	96.42 ± 20.97
VB ultimate load (N)	0	16.81 ± 3.22	35.41 ± 6.72	30.92 ± 5.16
	25	20.76 ± 4.76	33.19 ± 4.37	26.33 ± 0.2.02
	50	15.55 ± 3.82	28.91 ± 2.91	26.21 ± 1.46**
	100	12.80 ± 1.77	29.32 ± 2.95	18.20 ± 4.30***
VB stiffness (N/mm)	0	57.16 ± 19.58	56.10 ± 16.27	77.81 ± 29.34
	25	44.61 ± 9.71	48.44 ± 12.49	49.30 ± 15.71
	50	42.83 ± 10.61	49.22 ± 12.22	61.30 ± 21.62
	100	29.34 ± 9.78**	49.76 ± 11.83	47.79 ± 24.79
FN ultimate load (N)	0	16.68 ± 3.46	22.97 ± 3.16	16.97 ± 1.08
	25	18.15 ± 1.86	19.55 ± 3.13	15.62 ± 0.56
	50	15.37 ± 1.77	22.61 ± 3.13	14.64 ± 1.70*
	100	12.80 ± 1.17**	20.80 ± 2.95	13.27 ± 2.69**
FN stiffness (N/mm)	0	43.72 ± 10.19	63.00 ± 15.75	53.66 ± 9.51
	25	48.36 ± 6.23	48.47 ± 9.20	51.69 ± 13.36
	50	36.51 ± 3.21	59.57 ± 14.34	47.84 ± 18.11
	100	40.22 ± 10.06	58.29 ± 14.55	53.00 ± 21.78

Values for femora and vertebral bodies mechanical properties determined by three-point bending test of the femora (femora ultimate load, femora stiffness), by vertebral bodies compression test (VB ultimate load, VB stiffness) and by femoral neck-fracture test (FN ultimate load, FN stiffness). Data are presented as means ± SD. A p value <0.001 is noted as ***. A $0.001<p<0.05$ is noted as ** and a $0.05<p<0.1$ is noted as *. The fluoride treatment altered the bone mechanical properties in the "susceptible" A/J strain and in the "intermediate" SWR/J strain, with a more profound effect in the A/J strain.

Vertebral compression test

Seventy-five vertebral compression tests were successfully performed. The comparison of the treatment groups within the same strain showed significant differences in the A/J and SWR/J strains. No significant differences were noticed in the 129P3/J strain (Table 2).

In A/J strain, we found significant decreases in stiffness with increasing fluoride dose treatment. There was a significant difference between the treatment group 0 ppm and 100 ppm ($p=0.003$). In SWR/J, we found a significant progressive decrease in ultimate load with increasing fluoride dose treatment. There was a significant difference between the treatment group 0 ppm and the treatment group 100 ppm ($p<0.001$).

Femoral neck-fracture test

Seventy-four femoral neck-fracture tests were successfully performed. The comparison of the treatment groups within the same strain showed significant differences in the strains A/J and SWR/J. No significant differences were noticed in the strain 129P3/J, among all the F doses (Table 2). In the A/J strain, there was a decrease in ultimate load with increasing fluoride dose treatment, with significant differences between the treatment group 0 ppm and the treatment group 100 ppm ($p=0.017$). In SWR/J, there was a decrease in ultimate load with increasing fluoride doses, with a significant difference between the treatment group 0 ppm and the treatment group 100 ppm ($p=0.003$) (Table 2).

Discussion

Several studies in the literature [11,13–17] suggest that genetic determinants may influence an individual's susceptibility or resistance to fluoride effects. However, most of the studies are from epidemiological cohorts and, to our knowledge, no animal study has been published regarding the genetic resistance/susceptibility to fluoride effect on bone. In this study, inbred mice strains were used. Each strain represents a genetically homogeneous group. Isogenicity within an inbred strain and the genetic heterogenicity between inbred strains have allowed us to study the genetic contribution of fluoride on bone.

The fluoride concentrations in the drinking water delivered to the mice are well beyond what humans will ingest from fluoridated water. When drinking water is optimally fluoridated, corresponding plasma fluoride concentration ranges from 0.5 to 1.5 μmol/l [22]. These plasma concentrations are not likely to affect bone cells. But the therapeutic range of fluoride for osteoporosis treatment is regarded as between 5.0 and 10.0 μmol/l. In our study, we selected [F] in the water that would yield serum concentrations of ~8 μmol/l at 50 ppm and ~12 μmol/l at 100 ppm.

The analysis of the F concentrations in the bones demonstrated that, at baseline 0 ppm, there was more fluoride in the bones of 129P3/J mice. This indicates that at low F exposure genetic background can influence F accumulation in bone. The mechanism(s) responsible are not known but may involve potential differences in renal function or bone turnover in 129P3/J mice. At 25 ppm and 50 ppm treatment, SWR/J mice accumulated more fluoride than A/J or 129P3/J, indicating again that genetic factors may influence the fluoride accumulation in bone. However, at 100 ppm treatment, there were no differences in bone [F] between the 3 strains.

Although each strain showed a significant dose-dependent increase of fluoride in femora and vertebral bodies, the fluoride treatments neither affected bone geometry nor bone mineral density in the 3 strains. It has been shown that fluoride treatment increases the mineralization of bone in humans [23], rats [24], rabbits [25] or chickens [26]. In this study, we did not observe an effect of fluoride on BMD within the same strain, regardless of fluoride dose. This cannot be explained by low bone fluoride concentrations as we demonstrated that there was a dose-dependent increase of fluoride in femora and vertebral bodies. The fact that the mice were euthanized when they were still growing, even though at a lower rate, might play a role in this observation.

Bone mechanical properties varied depending upon genetic background and F dose. Three-point bending tests of the femora demonstrated that fluoride treatment altered the bone mechanical properties in the A/J strain only. Vertebral body compression tests and femoral neck-fracture tests demonstrated that fluoride treatment altered the bone mechanical properties in both A/J and SWR/J strains. As fluoride neither influenced the bone gross morphology nor the BMD in the three strains, these mechanical changes cannot be explained by alterations of the bone macroarchitecture or BMD. The fluoride treatment may have altered the "bone quality", i.e. bone material properties, in A/J and SWR/J strains.

Three-point bending results did not show any influence of the fluoride treatments on bone mechanical properties in SWR/J strain, although this strain showed significant mechanical alterations in the vertebral body compression test. This can be explained by the fact that genetic backgrounds may influence the mechanical behavior of cortical (femur) and trabecular (vertebrae) bone differently. Several studies have demonstrated that cortical and trabecular bone may be differently regulated [27–29].

Recent studies have emphasized the influence of an individual's genetic background in the efficacy and potential for adverse effects to some medications [30–39]. These genetic factors can be explained by differences in drug concentration, i.e. genetic variations in drug absorption, drug metabolism and drug transporters, as well as by differences in drug interactions with its receptors [32,40–42]. In our study, the three mechanical tests demonstrated that the fluoride treatment significantly altered the mechanical properties in the AJ strain, which was known to be a "susceptible" strain. The fluoride treatment also partially altered the mechanical

properties in the SWR/J strain, which is an "intermediate" strain for dental fluorosis. The fluoride treatment did not influence the bone quality in the 129P3/J strain, which is a "resistant" strain. These results could not be explained by different fluoride concentrations in bones since the SWR/J strain accumulated more fluoride at 25 ppm and 50 ppm treatment, and no significant difference was noticed at 100 ppm between the 3 strains. Therefore, genetic variation in fluoride uptake and excretion cannot explain the differences in mechanical properties observed between the 3 strains. These differential responses may be explained by different mechanisms which may be associated. Different physicochemical reactions creating the fluoroapatite crystals may be a plausible explanation as we know that such crystals normally affect bone quality. We do not know if crystalline structure differs between the 3 strains. Differences in bone geometry may play a role too. Bone geometry was shown to be slightly different between the 3 strains and initial bone strength differed as well. At 0 ppm treatment, 129P3/J mice had stronger bones and BMD was higher in these mice (Tables 1 and 2). Another possible explanation to the differential responses to fluoride would be an effect of fluoride on bone cells. Some studies [43,44] suggest that high bone mass can result from several factors including higher osteoblast activity. It is worth pointing out that mice with high bone mass are not great responders to anabolic agents. There is growing evidence that the sensitivity of trabecular tissue to both anabolic and catabolic stimuli is influenced by genetic background and may explain in part why various prophylactic treatments for low bone mass are not effective in everyone [45]. Therefore, a possible explanation could be a difference in bone adaptation responses which would be greater in AJ strain than in 129P3/J. Such differences in bone adaptation responses have already been demonstrated in some studies on mice [46,47]. But we still do not know how these can lead to different effects on bone quality. Selenium, another trace element, is known to have genotoxic effect at high levels [48]. Such interaction between a trace element and genes might be another mechanism explaining the genetic influence on fluoride bone effect.

In conclusion, the fluoride treatment altered the bone mechanical properties in the "susceptible" A/J strain and in the "intermediate" SWR/J strain, with a more profound effect in the A/J strain. The way the genetic background influences the fluoride bone effect is still unclear. Possible explanations are differences in crystallinity, different effects of fluoride on bone cells leading to differences in bone adaptation responses and/or genotoxic effect of fluoride. Study on the effect of different fluoride dose treatments on bone microarchitecture and mineralization in these different inbred strains of mice is in progress. The genetic influence on the efficacy and adverse effects has been demonstrated for some medications but has never been demonstrated for bone response to fluoride. The demonstration of such genetic influence on bone response to fluoride has important clinical significance. It stresses the importance to taking into account the genetic background of each individual.

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Fluoride effects on bone formation and mineralization are influenced by genetics

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ABSTRACT

Introduction: A variation in bone response to fluoride (F^-) exposure has been attributed to genetic factors. Increasing fluoride doses (0 ppm, 25 ppm, 50 ppm, 100 ppm) for three inbred mouse strains with different susceptibilities to developing dental enamel fluorosis (A/J, a "susceptible" strain; SWR/J, an "intermediate" strain; 129P3/J, a "resistant" strain) had different effects on their cortical and trabecular bone mechanical properties. In this paper, the structural and material properties of the bone were evaluated to explain the previously observed changes in mechanical properties.

Materials and methods: This study assessed the effect of increasing fluoride doses on the bone formation, microarchitecture, mineralization and microhardness of the A/J, SWR/J and 129P3/J mouse strains. Bone microarchitecture was quantified with microcomputed tomography and strut analysis. Bone formation was evaluated by static histomorphometry. Bone mineralization was quantified with backscattered electron (BSE) imaging and powder X-ray diffraction. Microhardness measurements were taken from the vertebral bodies (cortical and trabecular bones) and the cortex of the distal femur.

Results: Fluoride treatment had no significant effect on bone microarchitecture for any of the strains. All three strains demonstrated a significant increase in osteoid formation at the largest fluoride dose. Vertebral body trabecular bone BSE imaging revealed significantly decreased mineralization heterogeneity in the SWR/J strain at 50 ppm and 100 ppm F^-. The trabecular and cortical bone mineralization profiles showed a non-significant shift towards higher mineralization with increasing F^- dose in the three strains. Powder X-ray diffraction showed significantly smaller crystals for the 129P3/J strain, and increased crystal width with increasing F^- dose for all strains. There was no effect of F^- on trabecular and cortical bone microhardness.

Conclusion: Fluoride treatment had no significant effect on bone microarchitecture in these three strains. The increased osteoid formation and decreased mineralization heterogeneity support the theory that F^- delays mineralization of new bone. The increasing crystal width with increasing F^- dose confirms earlier results and correlates with most of the decreased mechanical properties. An increase in bone F^- may affect the mineral–organic interfacial bonding and/or bone matrix proteins, interfering with bone crystal growth inhibition on the crystallite faces as well as bonding between the mineral and organic interface. The smaller bone crystallites of the 129P3/J (resistant) strain may indicate a stronger organic/inorganic interface, reducing crystallite growth rate and increasing interfacial mechanical strength.

Introduction

Fluoride (F^-) is a trace element that is incorporated into bone mineral during bone formation [1]. Fluoride substitutes for the hydroxyl group in hydroxyapatite, forming fluorapatite. The action of fluoride on bone has been extensively studied and this ion has been shown to have an effect on bone mineral, bone cells and bone architecture [1]. The dose-dependent effects on mechanical properties are well-known, but the underlying mechanisms are still not fully understood. Furthermore, dose level alone is not the only factor affecting bone quality; the role of genetic factors has been emphasized in some epidemiological and clinical studies. Previous studies provide evidence of human non-responder populations to F^- [2] while some populations seem to be very sensitive to F^- at a wide range of doses [3–7]. Three inbred strains of mice (A/J, SWR/J, 129P3/J) that displayed variations in the onset and severity of dental/enamel fluorosis with

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equivalent F^- exposure [8], were shown to have different reductions in bone mechanical properties with equivalent F^- concentrations in their mineralized tissues [9]. The bone mechanical properties were reduced in the "susceptible strain" (A/J), moderately altered in the "intermediate strain" (SWR/J) and unaffected in the "resistant strain" (129P3/J), suggesting a genetic contribution to the variation in bone response to F^- content.

The purpose of this study was to assess the effects of increasing F^- doses on bone properties at the microscopic level (architecture, geometry, histology) and on bone mineralization, crystallite size and microhardness for the A/J, SWR/J and 129P3/J mouse strains in order to determine a possible mechanism for the previously reported differences in bone mechanical properties caused by F^- exposure.

Materials and methods

Animal husbandry

Mice

The bones used in this study were obtained from mice used for a previous study [9]. These were weanling (3-week-old) male mice from A/J, SWR/J, and 129P3/J inbred strains obtained from The Jackson Laboratory (Bar Harbor, ME). These three strains were selected because of differences in susceptibility to develop dental fluorosis (A/J is a "susceptible" strain, SWR/J is an "intermediate" strain and 129P3/J is a "resistant" strain) [8] as well as a varied response of bone mechanical properties to fluoride exposure [9]. This study was approved by the Indiana University School of Dentistry IACUC and all mice were housed within the Indiana University School of Dentistry Bioresearch Facility, an AAALAC-accredited unit. Mice were contained in boxed caging and allowed food and water *ad libitum*. They were fed a laboratory rodent diet (#5001, PMI® Nutrition International, Richmond, IN) that contained, by dry weight, 0.95% calcium, 0.67% phosphorus, 4.5 IU/g vitamin D3, and 18 ppm fluoride.

Fluoride treatment

The deionized water was tested from multiple sites within the IUSD Bioresearch Facility and showed [*F*] ion = 0.02 + 0.01 ppm. NaF was used to raise the [*F*] ion to 25 ppm, 50 ppm and 100 ppm. Four levels of fluoride (0, 25, 50 and 100 ppm F^-) were thus delivered in the drinking water as NaF from 3 weeks of age, for a period of 42 days. Deionized and fluoridated water was periodically analyzed by ion specific electrode (ISE) for fluoride concentration. Each batch of prepared 25 ppm, 50 ppm, and 100 ppm water for the study was used only if the fluoride was within 4% of the target concentration. Each treatment group consisted of 6 mice, except for the A/J (25 ppm), A/J (50 ppm) and 129P3/J (100 ppm) groups which consisted of 7 mice. At the end of the F^- treatment, the mice were humanely euthanized. Selected bones were harvested for analysis.

Design of experiment

Preparation of bones

The lumbar vertebral bodies (VB) and femora (F) were dissected and kept moist with saline solution during preparation. They were stored at −20 °C.

Strut analysis

Distal thoracic vertebral bodies were used for strut analysis. Each vertebra was cleaned and trimmed to leave only the vertebral body. They were fixed in 10% neutral buffered formalin for 1 week. Samples were then dehydrated in ascending concentrations of acetone and subsequently infiltrated in ascending ratios of unpolymerized Spurr resin and acetone. The bones were finally embedded in blocks of Spurr resin that was polymerized in a 60 °C oven for 48 h. Several 5-micron thick coronal sections were then cut from each sample, using an automatic Reichert-Jung 2500 rotary microtome (Leica Microsystems). Sections were placed on gelatinized slides and incubated in a 60 °C oven for 48 h, then stained with Von Kossa and imaged using light microscopy. Trabecular bone area was analyzed using the Quantimet 570 (Leica) image processing and analysis system. The connectivity was evaluated by skeletonizing the image of trabecular bone, representing the trabeculae as struts. Nodes represented points at which three or more struts were connected. The number of nodes (NN; mm^{-2}) was determined as a measure of connectivity, and the number of free ends (NFE; mm^{-2}) was determined as a measure of disconnectivity [10].

Microcomputed tomography of thoracic vertebral bodies

Thoracic vertebral bodies were scanned using microcomputed tomography (μCT) to evaluate the trabecular bone architecture (Fig. 1). These vertebrae were embedded in microtubes using epoxy resin to eliminate movement during the scan. μCT was performed using a desktop μCT with a 6.5 μm voxel size (GE Medical Systems eXplore Locus SP Specimen Scanner, London, Ontario, Canada). Scans were reconstructed and calibrated using a hydroxyapatite standard. Final images and 3D volumes were analyzed using MicroView CT visualization software (GE Medical Systems, London, Ontario, Canada). Each vertebral body was cropped out of the reconstructed volume and rotated so that the mediolateral axis coincided with the x-axis. The region of interest (trabecular bone) was then defined in each vertebral body 3D image and analyzed to determine the following trabecular bone parameters: bone volume fraction (BVF = Bone Volume / Total Volume; %), bone surface area (SA; mm^2), mean trabecular thickness (TbTh; μm), trabecular number (TbN; mm^{-1}), and mean trabecular separation (TbSp; μm). The structure model index (SMI) was also determined. SMI gives information about the curvature of the surface and estimates how "plate-like" or "rod-like" a trabecular structure is. A SMI value of zero indicates bone architecture with "plates" while a SMI value of 3 is an indicator of "rods". Anisotropy was analyzed by determining the ratio between the length of one axis versus another (a1/a3, a1/a2, a2/a3). This allowed us to determine the degree of symmetry and orientation of the trabecular structure: the closer the ratio is to 1, the less anisotropic the sample.

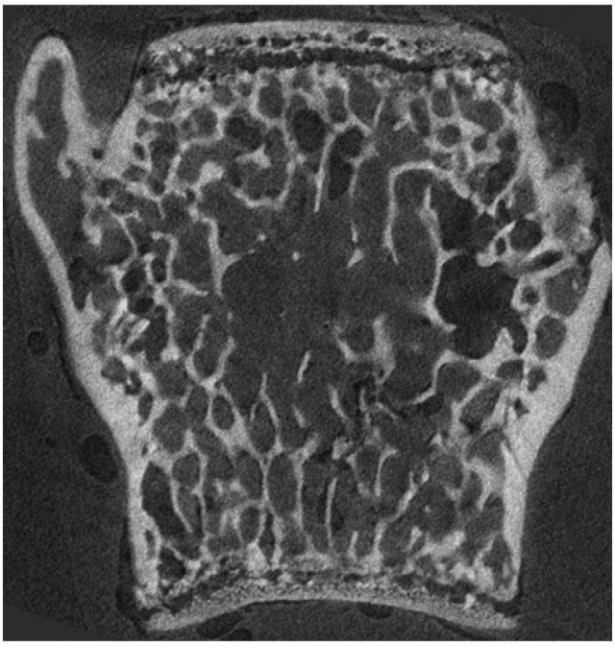

Fig. 1. Coronal section of a thoracic vertebral body, obtained with microcomputed tomography.

Static histomorphometry

Coronal sections at midpoint of the embedded vertebral bodies used for strut analysis were stained with Goldner's trichrome, resulting in blue/green staining of mineralized bone and red/orange staining of osteoid [11]. Trabecular bone was analyzed using a 25× objective lens (Zeiss) connected to a video camera (Retiga 1300). The total trabecular area was analyzed as serial fields using the Leitz Bioquant morphometry system (Bioquant Nova Prime, Version 6.50.10). We determined the following static histomorphometric parameters: trabecular bone volume (BV/TV; %; ratio between trabecular bone volume and tissue volume), mineralized trabecular bone volume (MdV/TV; %; ratio between mineralized trabecular bone volume and tissue volume), osteoid volume (OV/BV; %; ratio between osteoid volume and bone volume), osteoid surface (OS/BS; %; ratio between total osteoid surface and bone surface) and osteoid thickness (OTh; μm).

Backscattered electron imaging

Following three-point bending of right femora, as reported in a previous study [9], the distal end of the broken femur was embedded in Spurr blocks, using the same procedure as described for the vertebral bodies. The embedded femora as well as the Spurr-embedded blocks of thoracic vertebral bodies were used for evaluation of mineralization distribution using backscattered electron (BSE) imaging. Blocks were cut, polished, carbon-coated and imaged using backscattered electron (BSE) imaging (solid state BSE detector, FEI Company, Hillsboro, OR, USA) on a Philips XL30 ESEM (FEI). The relative backscattering of the mineralized tissues was determined by comparison with a silicon dioxide standard, which was measured between every specimen. Histograms of the grey level distribution were created for the trabecular bone of the vertebral bodies and for the distal femur cortex. Increasing brightness corresponds with increasing mineralization [12]. From the histogram, the grey level of the histogram peak was determined and used to represent the overall degree of mineralization. The full width at half the maximum height (FWHM) of the histogram represents the heterogeneity of the mineralization distribution, and gives an approximation of the distribution of less-mineralized (younger) and more mineralized (older) bone.

Powder X-ray diffraction/apatite crystallite size

Powder X-ray diffraction was performed on bone powder, produced from left femora that were previously used to determine femur fluoride concentration [9]. The powder was prepared as follows: Bones were manually crushed, tri-washed and lyophilized for 48 h. A 2:1 chloroform:methanol solution was used to defat the specimens overnight [13]. The solution was decanted and replaced with methanol. After one hour, the methanol was decanted and the specimens dried at ambient temperature. Dry specimens were ground to a powder (<45 μm particle size) using a cryogenic freezer mill (SPEX Certiprep 6750 Freezer Mill).

The bone powder specimens and a NIST standard reference material (2910, Calcium Hydroxyapatite) were scanned from 24.5 to 27.0 °2θ at a scan speed of 0.1 °2θ/min and 37.0 to 42.0 °2θ at a scan speed of 0.05 °2θ/min (step size 0.004 °2θ, 40 kV, 30 mA, Cu Kα radiation, Rigaku MultiFlex, Rigaku/MSC, The Woodlands, TX, USA). All specimens were remounted and re-scanned for a total of 3 scans/specimen. Crystallite size was calculated from the peak broadening of the powder X-ray diffraction peaks [14].

Peak broadening is quantified with the "full width at half the maximum height" (FWHM) of the peak ($\beta_{1/2}$), which is composed of broadening caused by both the specimen and the instrument. The FWHM of the 26 °2θ peak ((002) plane-hydroxyapatite crystal length) and 40 °2θ peak ((310) plane-hydroxyapatite crystal cross-section or width) were quantified with the profile fitting function of Jade (XRD pattern processing software, v. 6.5, Materials Data Inc.). The FWHM due to the instrument (instrument broadening (β_i)), was measured by scanning reference silicon at 26 and 55 °2θ. The broadening due to the specimen only (corrected $\beta_{1/2}$) was calculated from the square root of the instrument broadening squared subtracted from the measured specimen peak broadening squared. This corrected $\beta_{1/2}$ was used to calculate the crystalline size between the (002) and the (310) planes using the Scherrer equation,

$$D = \frac{57.3K\lambda}{\beta_{1/2}\cos(\theta)}$$

where 57.3 is a conversion factor from degrees to radians, K is a correction factor (0.9) used to reflect the elongated apatite crystals of bone, λ is the K-emission wavelength of copper, θ is the diffraction angle, and D is the size of the crystallite in angstroms along the specific axis. The means of three independent FWHM values for each 26 and 40 °2θ peak were calculated for each specimen.

Cortical and trabecular microhardness

After the BSE study was complete, microhardness measurements of the Spurr-embedded thoracic vertebral bodies and distal femora were performed. The microhardness of trabecular bone (coronal surface of vertebral bodies) and cortical bone (coronal plane of vertebral body cortical shell and axial surface of distal femora) was determined by using a microhardness testing machine (Mitutoyo HM-122, S/N 260113). Microhardness testing consists of measuring the resistance of bone to indentation. During the test, a pyramidal diamond indenter of known geometry was lowered onto the sample under a known load for 10 s, leaving an indentation on its surface. The depth of the indentation is related to the hardness of the bone. Given that the diamond is pyramidal, there is a linear relationship between the length of the indentation and the depth. Length can therefore be used as a proxy measure for depth. The indentation length and the load are used to calculate the hardness of the bone, using this equation:

$$\mathrm{HV} = k\frac{F}{S} = 0.102\frac{F}{S} = 0.102\frac{2F\sin\frac{\theta}{2}}{d^2} = 0.1891\frac{F}{D^2}$$

where HV is the Vickers Hardness, k is a constant ($k = \frac{1}{g} = \frac{1}{9.8} \cong 0.102$, where g is the standard acceleration due to gravity), F is the test force (0.025 kg), S is the surface area of indentation (mm^2), d is the average length of two diagonals (mm), and θ is the face-to-face apex angle of diamond indenter (136°). Ten measures were performed for each type of bone (trabecular/cortical) for each sample and the average of these ten measures was calculated.

Statistical analysis

Statistical analysis was performed using SPSS (version 12.0) (SPSS Inc., Chicago IL) software. Two-way Analysis of Variance (ANOVA, general linear model) was used to compare the effects of F^- treatment and genetic strain on the bone properties. Post hoc multiple comparisons between the three strains and four fluoride treatments were performed using the Bonferroni test. The correlation between the crystallite width and mechanical properties for each strain was made with the bivariate correlation function (SPSS) with a two-tailed Pearson coefficient. A p value of <0.05 was required to consider a difference significant. A confidence level of 90% ($p<0.1$) indicated a statistical trend. Data are presented as mean±SD.

Results

Strut analysis

There were no significant changes in the thoracic VB trabecular bone connectivity with fluoride treatment in the three strains (Table 1).

Table 1
Evaluation of thoracic vertebral body trabecular bone connectivity by strut analysis

	Fluoride treatment (ppm)	Strain A/J	Strain SWR/J	Strain 129P3/J
NFE	0	39.67±7.24	42.51±12.83	38.05±8.41
	100	39.25±8.55	50.88±7.09	47.08±7.39
NN	0	10.24±3.26	20.76±3.80	25.36±9.24
	100	7.25±2.26	14.87±5.71	24.36±4.29
BVF (%)	0	0.22±0.03	0.27±0.03	0.30±0.03
	100	0.20±0.02	0.26±0.02	0.27±0.02
SA (mm^2)	0	23.58±2.94	27.68±4.42	41.08±4.18
	100	23.64±6.45	28.63±3.88	35.25±7.22
TbTh (μm)	0	0.026±0.002	0.026±0.004	0.028±0.003
	100	0.024±0.003	0.025±0.002	0.027±0.002
TbN (mm^{-1})	0	8.66±1.19	10.03±0.98	10.66±0.43
	100	8.55±1.55	10.22±0.83	9.92±1.20
TbSp (μm)	0	0.091±0.014	0.074±0.008	0.066±0.003
	100	0.097±0.021	0.073±0.007	0.075±0.012
SMI	0	1.06±0.40	0.47±0.57	0.37±0.35
	100	1.06±0.38	0.69±0.22	0.48±0.28
a1/a3	0	1.73±0.08	1.46±0.07	1.52±0.04
	100	1.51±0.20	1.45±0.09	1.55±0.13
a1/a2	0	1.53±0.07	1.37±0.05	1.41±0.06
	100	1.32±0.17	1.35±0.03	1.38±0.13
a2/a3	0	1.13±0.07	1.07±0.04	1.08±0.04
	100	1.14±0.05	1.07±0.05	1.12±0.06

Analysis of the number of free ends (NFE, disconnectivity) and of the number of nodes (NN, connectivity).
Trabecular bone parameters (BVF, SA, TbTh, TbN and TbSp), SMI and anisotropy analysis of the thoracic vertebral body in the three strains.
Data are presented as means±SD. No significant statistical differences were noted.

Table 2
Histomorphometric analysis of the thoracic vertebral body in the three strains

	Fluoride treatment (ppm)	Strain A/J	Strain SWR/J	Strain 129P3/J
% Bone volume of tissue volume BV/TV	0	11.48±2.86	19.83±3.15	24.65±8.66
	25	12.55±3.11	18.36±3.54	24.50±4.50
	50	13.58±2.64	20.46±4.72	23.69±6.93
	100	12.85±1.62	15.44±2.89	24.43±2.97
% Mineralized bone of tissue volume MdV/TV	0	11.47±2.84	19.83±3.15	24.62±8.64
	25	12.54±3.11	18.35±3.53	24.49±4.50
	50	13.58±2.64	20.45±4.72	23.64±6.93
	100	12.81±1.62	15.43±2.89	24.27±2.94
% Osteoid volume of bone volume OV/BV	0	0.01±0.01	0.01±0.02	0.11±0.07
	25	0.02±0.03	0.05±0.05	0.05±0.03
	50	0.04±0.04	0.03±0.03	0.20±0.07
	100	0.26±0.05**	0.07±0.03	0.64±0.32***
% Osteoid surface (OS/BS)	0	0.09±0.19	0.22±0.39	2.51±1.65
	25	0.32±0.45	0.54±0.69	1.04±0.83
	50	0.67±0.59	0.40±0.25	4.05±1.18
	100	4.10±0.91***	1.20±0.54*	9.40±3.48***
Osteoid thickness (mcm) OTh	0	0.38±0.52	0.68±0.76	1.24±0.28
	25	0.31±0.49	0.90±0.72	1.16±0.13
	50	0.94±0.66	0.95±0.53	1.40±0.12
	100	1.37±0.10	1.38±0.25	1.61±0.27**

Data are presented as means±SD. A p value <0.001 is noted as ***. A $0.001<p<0.05$ is noted as ** and a $0.05<p<0.1$ is noted as *. No significant statistical differences were noted for the BV/TV and mdV/TV, but there were significant differences in osteoid formation in the three strains.

Microcomputed tomography of thoracic vertebral bodies

Analysis of the trabecular bone parameters (BVF, SA, TbTh, TbN and TbSp) did not show any significant differences between the groups (Table 1). No statistical differences were observed between SMI values (Table 1). Anisotropy analysis (a1/a3, a1/a2, a2/a3) did not show any significant change with fluoride treatment within the three strains (Table 1).

Static histomorphometry

The only significant histomorphometric differences observed were in osteoid formation (Table 2). There was an increase in osteoid volume and osteoid surface between the control and the 100 ppm groups for all three strains. The increase observed in osteoid thickness was statistically significant for the 129P3/J strain. The percent increase in osteoid volume for the three strains correlated with their susceptibility to dental fluorosis, with a 26-fold increase for the A/J strain, a 7-fold increase for the SWR/J and a 6-fold increase for the 129P3/J strain. The osteoid surface followed a similar trend, with a 46-fold increase for the A/J, a 5-fold for the SWR/J and a 4-fold increase for the 129P3/J strain. This trend also applied to osteoid thickness, with a 4-fold increase for the A/J, a 2-fold increase for the SWR/J and a 1.3-fold increase for the 129 P3/J strain. The comparison of the three strains for each fluoride dose treatment showed that the osteoid volume and surface were significantly larger in the 129P3/J strain ($p \leq 0.05$).

Backscattered electron imaging

The average peak grey level of femur cortical bone increased with fluoride treatment, but the observed differences were not statistically significant (Table 3). Grey levels increased by 2% in the A/J strain, by 8% in the SWR/J strain and by 12% in the 129P3/J strain at 100 ppm F^-. The FWHM did not demonstrate any significant changes in the three strains (Table 3).

While the average peak grey level of vertebral body trabecular bone increased with fluoride treatment, the observed differences were not statistically significant (Table 3). Grey level increased by 7% in A/J, by 20% in SWR/J and by 12% in 129P3/J (Table 3). While the FWHM decreased in the three strains with F^- treatment, these changes were only significant in the SWR/J (Table 3). The FWHM decreased by 4% in A/J, by 22% in SWR/J and by 21% in 129P3/J.

X-ray diffraction study

A 2-way ANOVA analysis of the effect of genotype and fluoride dose on the crystal length (002) showed an effect of genotype on crystallite length, independent of fluoride dose. The crystallite length of the resistant (129P3/J) strain was significantly smaller than the same dimension for the other strains ($p<0.05$) (Table 4). The F^- treatment did not significantly affect the crystal length in any of the three strains.

Table 3
BSE analysis of distal femur cortical bone and thoracic vertebral body trabecular bone

	Fluoride treatment (ppm)	A/J	SWR/J	129P3/J
BSE analysis of distal femur cortical bone				
Peak grey level	0	212±13	194±14	190±15
	25	200±22	191±12	215±13
	50	201±15	179±19	210±19
	100	216±2	210±12	212±13
FWHM	0	44±10	39±9	44±8
	25	36±5	42±7	39±3
	50	38±4	43±10	43±4
	100	46±3	46±4	45±1
BSE analysis of vertebral body trabecular bone				
Peak grey level	0	181±15	171±23	165±11
	25	170±20	179±24	171±33
	50	176±20	180±24	178±8
	100	193±14	206±18	185±16
FWHM	0	55±8	65±6	62±11
	25	57±9	61±6	62±11
	50	49±7	51±7***	53±8
	100	53±7	51±6***	48±1*

Data are presented as means±SD. A p value <0.001 is noted as ***. A $0.001<p<0.05$ is noted as ** and a $0.05<p<0.1$ is noted as *. For vertebral body trabecular bone, the fluoride dose significantly affected the full width of the half maximum value (FWHM) in the SWR/J strain.

Table 4
Bone mineral crystal length (002) and bone mineral crystal width (310), estimated by X-ray diffraction

	Fluoride Treatment (ppm)	A/J	SWR/J	129P3/J
Bone mineral crystal length (Å)	0	147.2±12.1	148.5±5.9	130.4±2.6
	25	140.3±4.4	143.6±9.4	135±6.8
	50	146.7±8.7	144.4±10.6	133.5±6.3
	100	141±3.5	148.4±10.5	133.9±7.0
Bone mineral crystal width (Å)	0	56.2±1.1	55.3±1.4	52.2±1.3
	25	56.2±1.0	56.4±1.1	53.5±1.5
	50	58.5± 1.8**	58.4±2.1	54.5±1.0***
	100	59.4±1.1***	61.2±2.2***	56.4±1.0***

Data are presented as means±SD. A p value <0.001 is noted as ***. A $0.001<p<0.05$ is noted as ** and a $0.05<p<0.1$ is noted as *. The fluoride treatment did not significantly affect the crystal length, but it induced a significant increase of the crystal width in all three strains. The crystal length and width were significantly smaller in the strain 129P3/J ($p<0.05$), compared to the other two strains.

A similar analysis for the crystallite width (310) showed an effect of both genotype and fluoride concentration, with no evidence of interaction between the two factors. The crystallite width of the 129P3/J strain was significantly smaller than for the A/J and SWR/J strains ($p<0.05$) (Table 4). All strains showed a significant increase in crystallite width between 0 and 100 ppm F^- (Table 4). The susceptible strain A/J showed a 5.7% increase in crystallite width, the intermediate strain SWR/J showed a 10.6% increase and the resistant strain 129P3/J showed a 8% increase. At 100 ppm F^- dose treatment, the crystallite width of the resistant strain 129P3/J was still significantly smaller than for the two other strains ($p<0.05$). The correlation analysis between the crystallite width and mechanical properties (from a previous study [9]) for each strain at all F^- dose treatments showed that the crystallite width increase correlated with most of the decreased mechanical properties described in the susceptible (A/J) and intermediate (SWR/J) strains (Table 5, Fig. 2).

Cortical and trabecular bone microhardness

No statistically significant differences were observed between the microhardness values of the cortical or the trabecular bone between the three strains at any F^- concentration (Table 6).

Discussion

In our previous study [9], we showed that increasing doses of fluoride (0 ppm, 25 ppm, 50 ppm, 100 ppm) had different effects on the bone properties of three inbred mouse strains which demonstrated different susceptibilities to developing enamel fluorosis (A/J, a "susceptible" strain; SWR/J, an "intermediate" strain; 129P3/J, a "resistant" strain). Although significant increases of fluoride concentration in femora and vertebral bodies could be demonstrated, and no significant effects on bone macroarchitecture and bone mineral densities (BMD) were found in the three strains, mechanical testing showed significant degradation of bone mechanical properties in the A/J strain, whereas moderate degradation in the SWR/J strain and no effect in the 129P3/J strain were observed. We concluded that genetic factors may contribute to the variation in bone response to fluoride exposure and that the differences observed between the three strains could not be explained by an alteration of the bone macroarchitecture or by a different influence of fluoride on BMD. The purpose of this study was to determine if microarchitecture, histomorphometry, mineralization or microhardness were influenced by fluoride treatments and could be correlated with the previously observed reduction in bone mechanical properties.

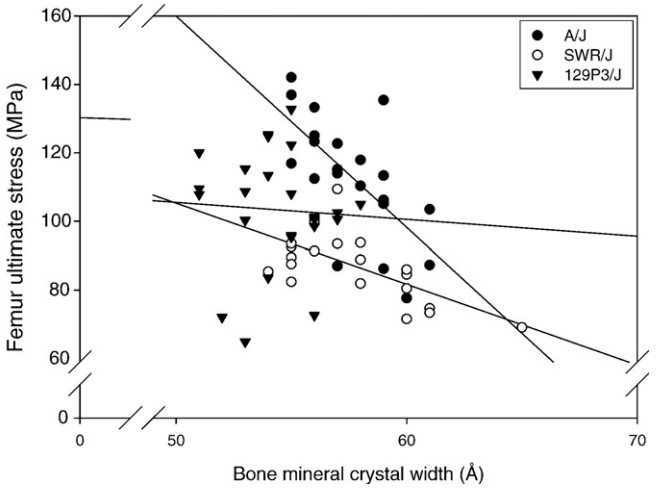

Fig. 2. Correlation analysis between the crystallite width and femur ultimate stress for each strain at all F^- dose treatments. Crystallite width increase correlates with the decreased femur ultimate stress observed in the susceptible (A/J) and intermediate (SWR/J) strains.

Regarding bone microarchitecture, neither microcomputed tomography of thoracic vertebral body nor strut analysis showed any significant changes with fluoride treatment in the three strains. Fluoride therapy has been showed to increase trabecular thickness but to leave connectivity unaltered [15]. In our study, we did not find any change in vertebral body connectivity or in trabecular thickness. The absence of significant changes may be explained by the fact that fluoride was administered for only 42 days, which may be too short a period of time to have any significant effect on trabecular thickness. Therefore the differences in bone mechanical properties observed for the A/J and SWR/J strains with fluoride treatment [9] cannot be explained by altered bone microarchitecture.

Table 5
Statistical significance of the effect of F^- dose on mechanical properties between the 0 and 100 ppm levels [9] and the Pearson correlation coefficient (r) between the bone crystallite width (HA width) and the mechanical property for all F^- doses (2-tailed significance)

	A/J		SWR/J		129P3/J	
	F^- dose	HA width	F^- dose	HA width	F^- dose	HA width
F ultimate load	$p<0.001$	$r=-0.70$***	NS	$r=-0.56$**	NS	NS
F stiffness	$0.001<p<0.05$	$r=-0.45$**	NS	$r=-0.40$*	NS	NS
F ultimate stress	$0.001<p<0.05$	$r=-0.67$**	$0.001<p<0.05$	$r=-0.65$**	NS	NS
F modulus	NS	NS	NS	$r=-0.47$**	NS	NS
VB ultimate load	NS	$r=-0.48$**	$p<0.001$	$r=-0.68$**	NS	NS
VB stiffness	$0.001<p<0.05$	$r=-0.40$*	NS	NS	NS	NS
VB ultimate stress	NS	$r=-0.46$*	$p<0.001$	$r=-0.82$***	NS	NS
VB modulus	$0.001<p<0.05$	$r=-0.39$*	NS	NS	NS	NS
FN ultimate load	$0.001<p<0.05$	$r=-0.72$***	$0.001<p<0.05$	$r=-0.61$**	NS	NS
FN stiffness	NS	$r=-0.50$**	NS	NS	NS	NS

NS: not statistically significant The crystallite width increase correlated with most of the decreased mechanical properties described in the susceptible (A/J) and intermediate (SWR/J) strains. A p value <0.001 is noted as ***. A $0.001<p<0.05$ is noted as ** and a $0.05<p<0.1$ is noted as *.

Table 6
Microhardness of trabecular and cortical bones in vertebral bodies (VB), and of cortical bone in distal femurs in each treatment group

	Fluoride treatment (ppm)	Strain A/J	Strain SWR/J	Strain 129P3/J
VB microhardness for trabecular bone	0	27.6±2.1	25.1±2.5	25.9±1.7
	100	24.5±2.4	24.8±3.2	25.6±1.7
VB microhardness for cortical bone	0	31.1±2.0	30.3±0.9	31.7±1.7
	100	31.4±3.1	30.6±1.6	31.4±1.3
Distal femur cortex microhardness	0	39.4±1.5	36.8±2.3	37.9±2.6
	100	38.0±2.5	35.3±1.0	37.5±1.7

Data are presented as means±SD. No statistical difference was noted for the microhardness of cortical and trabecular bones in the three strains.

It is recognized that fluoride is usually not incorporated into fully-mineralized, mature bone and accumulates only in bone formed during the period of fluoride exposure [16]. This explains why fluoride concentration is generally higher in sites with a high turnover rate, such as cancellous rather than cortical bone. In our study, fluoride was given to young (that is, rapidly growing) mice and this explains the significant increases (concordant with increasing fluoride dose) of fluoride concentration observed in femora and vertebral bodies in all three strains [9]. As noted in our previous paper, we selected fluoride concentration in the water that would yield serum concentrations of ~8 μmol/L at 50 ppm and ~12 μmol/L at 100 ppm, which are greater than the plasma fluoride concentrations measured in humans when drinking water is optimally fluoridated (0.5–1.5 μmol/L) [17], but which do correspond to the therapeutic range of fluoride for osteoporosis treatment (5–10 μmol/L). High doses of fluoride were shown to affect bone cells and therefore remodeling processes. Fluoride has been shown to have a mitogenic effect on osteoblasts [18–20]. Effects of fluoride on osteoclasts have also been described [21], and fluoride effects on osteoclastogenesis have been demonstrated to be influenced by genetic background [22]. The cumulative result of these effects is a net increase in bone formation. This is consistent with our observations of osteoid formation in all three mouse strains; the osteoid formation was the only parameter that showed significant changes. In all three strains, we noted a significant increase in osteoid formation at the largest fluoride dose. The percent increase in osteoid volume, surface and thickness for the three strains correlated with their susceptibility to dental fluorosis, but the overall osteoid formation was significantly larger in the 129P3/J strain.

Fluoride has also been shown to have an effect on mineralization. It retards mineralization and the mineral produced is less susceptible to dissolution [23–25]. Alterations in bone crystal structure have also been described [24]. In our study, the BSE mineralization profile of cortical and trabecular bones showed a non-significant increase towards higher mineralization with increasing F^- dose in the three strains. This is consistent with the increased resistance to dissolution of bone mineral with an increased fluoride content. Fluoride treatment had different effects on mineralization heterogeneity of the trabecular and cortical bones. We observed a decrease in mineralization heterogeneity in the trabecular bone, which was consistent with observations that fluoride delays mineralization of new bone. The absence of an effect on cortical bone mineralization heterogeneity may be explained by site-specific differences in bone turnover, as there is a higher turnover in trabecular bone.

It remains unclear if the substitution of fluoride ions for hydroxyl ions affects the size of the crystallites. Most authors accept that the crystal length is unchanged in F^- treatment [26,27]; however there remains debate about changes in crystal width. Some authors described an increase in crystal width [26,28,29], while others found no significant changes [27]. In this study, we observed a significant increase in crystal width at the largest F^- dose for all three strains. This relative increase in crystal width was similar in the three strains. However the absolute crystal size (length and width) was also inherently different between the strains. The 129P3/J ("resistant") strain had smaller crystals in both measured dimensions than the other strains for all fluoride doses. The overall smaller crystal size suggests that the crystal growth rate of the resistant strain is slower than that of the other strains. This reduced crystal growth rate may be due to different mineral–collagen interface conditions that reduce the flux of calcium and phosphate ions to the apatite crystal surface. We found a correlation between the crystallite width increase and most of the observed decreased mechanical properties in the A/J and SWR/J strains (Table 5, Fig. 2). These observations suggest that bone crystal size may play an important role in determining bone quality. The fact that the 129P3/J resistant strain has the strongest bone and the smallest bone crystal may suggest that these mice have a stronger mineral–organic interfacial bonding, which has previously been shown to play an important role in bone quality [30]. The quality of the mineral–organic interfacial bonding and/or bone matrix proteins could therefore be influenced by genetics, and the observed

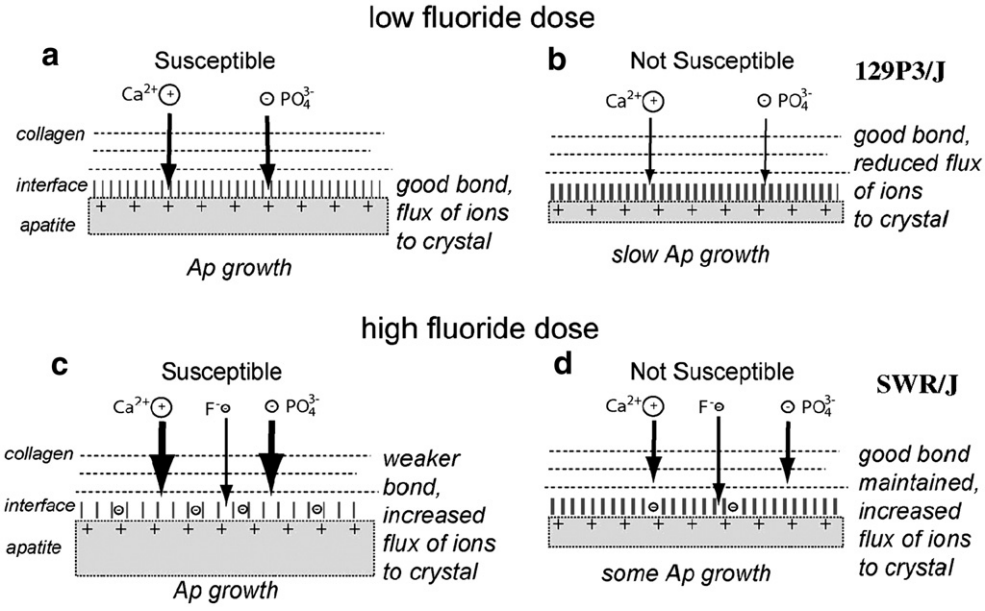

Fig. 3. Schematic of the effect of mineral–collagen interface quality and fluoride concentration on crystal growth and composite material strength.

differences between the three strains may be explained by different genetic factors whose specific action is still not known.

To test bone mechanical properties at the microscopic level, we performed a microhardness study of trabecular bone (thoracic vertebral body) and cortical bone (thoracic vertebral body and distal femur). This test has the theoretical advantage of eliminating architectural considerations, instead focusing on the tissue-level mechanical properties of the bone. In our study, we did not find statistically significant F^- induced changes in trabecular and cortical bone microhardness for any of the three strains. These results may be influenced by the lack of precision of this test. Variations in trabecular shape, orientation and number, as well as variations in mineral distribution and properties throughout individual trabeculae, are known to influence the results of this test [31]. This lack of precision is also present in cortical bone microhardness study, as we tested femur and VB cortical bone microhardness at one cross-section only. The absence of any change in microhardness may also be explained by the short period of fluoride treatment and by the fact that F^- was given to rapidly growing mice. Therefore we cannot draw any certain conclusions from the absence of significant differences in the results.

In conclusion, fluoride is known to have biological effects on bone cells, as well as chemical and physical effects on bone crystals. In the present study, fluoride treatment had no effect on bone microarchitecture in the three strains. The increased osteoid formation and decreased mineralization heterogeneity support the theories that F^- stimulates osteoblastic activity and delays mineralization of new bone. The 129P3/J resistant strain was shown to have smaller bone crystals and a correlation was found between the F^- induced increase in crystal width and most of the decreased mechanical properties. Interfacial bonding interactions between the mineral and organic phases of bone have been shown to play an important role in bone mechanical properties [32], and different types of interaction have been described [33–37]. In Fig. 3, we propose a model that could explain the effect of mineral–collagen interface quality and fluoride concentration on crystal growth and composite material strength. At low fluoride dose (Figs. 3a and b), the 129P3/J resistant strain has a stronger organic–inorganic interface than the strains A/J and SWR/J. This strong interfacial bonding may reduce crystallite growth rate, explaining the smaller bone crystals discovered in the 129P3/J strain, and may increase the composite material strength. *In vitro* studies have demonstrated that fluoride ions could alter bone mechanical properties by altering mineral–organic interfacial bonding [32,38,39]. At high fluoride dose (Figs. 3c and d), such interfacial bonding alteration could weaken the mineral–organic bond and reduce interfacial mechanical strength. The weaker mineral–organic interface allows an increased flux of calcium and phosphate ions to the apatite crystal surface, which explains the increased crystal width observed in the three strains. This increase in crystal width was similar in the three strains and the resistant 129P3/J strain had still the smallest bone crystals at high fluoride dose, which may explain the absence of bone mechanical properties alteration in this strain.

Another possible mechanism of fluoride action would be an alteration of bone matrix proteins, i.e. collagen and noncollagenous proteins. Collagen and noncollagenous proteins are of significant importance for the biomechanical integrity of the bone [31,40–43] and many bone matrix proteins play important roles in mineralization [44]. Miao et al. [45] have investigated the effects of fluoride treatment (221 mg/L NaF in drinking water for 2 months) on rat calvarial osteoblasts. They showed that excessive fluoride intake could inhibit the synthesis of type I collagen and decrease the degree of collagen cross-linking. An influence of fluoride on proteoglycan structure synthesized by mineralizing bone cells [46] and on expression of matrix metalloproteinases [47] has also been proposed. Kindt et al. [39] showed that enhanced formation of superficial fluorapatite weakened the protein-hydroxyapatite interfaces but NaF was also found to be a potent agent for extracting noncollagenous proteins from bone powder. It must be pointed out that these effects were demonstrated in *in vitro* studies and that they may be different *in vivo*.

Bone quality is thus a complex property that we do not fully understand, and the way the fluoride alters bone quality is still unclear. Possible mechanisms would be an alteration of mineral–organic interfacial bonding and/or an effect on bone matrix proteins. The present study also emphasizes the importance of considering bone mineral crystal size, mineral–organic interfacial bonding and bone matrix proteins in understanding bone quality. This has an important clinical impact, as alterations in bone collagen quality have been observed with aging [48].

Acknowledgments

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CHAPTER 4: DISCUSSION

4.1. Discussion of hypotheses

Hypothesis and objectives were exposed in details in the first part of this thesis. In this chapter, each point will be discussed in the light of the results obtained.

Regarding the F^- doses used in this study, it must be pointed out that the F^- concentrations in the drinking water delivered to the mice are well beyond what humans will ingest from fluoridated water. When drinking water is optimally fluoridated, corresponding plasma fluoride concentration ranges from 0.5 to 1.5 micromole/l [221]. These plasma concentrations are not likely to affect bone cells. But the therapeutic range of F^- for osteoporosis treatment is regarded as between 5.0 and 10.0 micromole/l. In this study, we selected F^- concentrations in the water that would yield serum concentrations of ~ 8 micromole/l at 50ppm and ~ 12 micromole/l at 100ppm.

Hypothesis 1: The fluoride concentration in bone will be the same for every mouse in a same treatment group, whatever the strain.

The analysis of the F^- concentrations in femora and vertebral bodies demonstrated that at baseline 0ppm, there was more F^- in the bones of 129P3/J mice ("resistant" strain). This indicates that at low F^- exposure genetic background can

influence F^- accumulation in bone. The mechanism(s) responsible is (are) not known but may involve potential differences in renal function or bone turnover between the three strains.

Concordant with increasing F^- dose in delivered water were significant increases of F^- concentration in femora and vertebral bodies from all three strains (Figure 15).

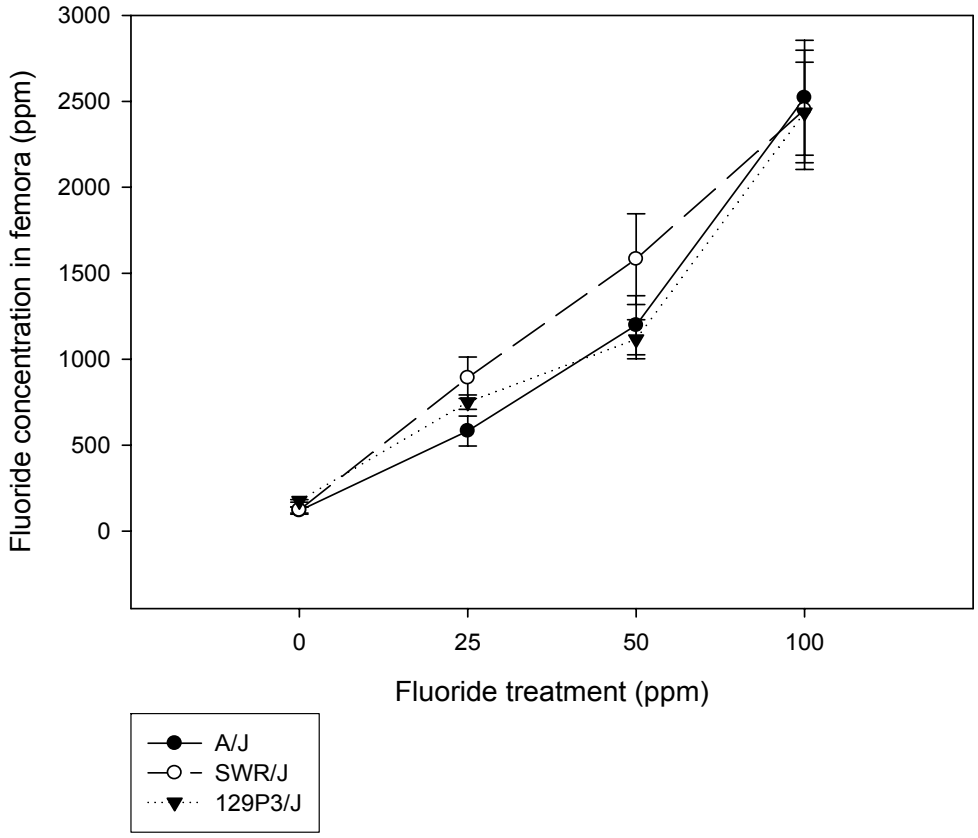

Figure 15: Fluoride (ppm) concentration in femora in each treatment group. Data are presented as means ± SD.

At 25ppm and 50ppm treatment, SWR/J mice ("intermediate" strain) accumulated more F^- than A/J ("susceptible") or 129P3/J, indicating again that genetic factors may

influence the F^- accumulation in bone. Although the SWR/J strain accumulated F^- moderately faster than the two other strains, all three strains had comparable F^- concentrations in femora and vertebral bodies at 100ppm treatment. This observation is concordant with the results described by Everett *et al.* [189] who showed that there was a significant correlation between F^- concentration in water and F^- concentration in femur but that the mice treated with the same F^- dose accumulated similar amounts of F^- in their femora whatever the strain considered. It is recognized that F^- is usually not incorporated into fully-mineralized, mature bone and accumulates only in bone formed during the period of F^- exposure [119, 222]. This explains why F^- concentration is generally higher in sites with a high turnover rate, such as trabecular rather than cortical bone. In our study, F^- was given to young (that is, rapidly growing) mice and this explains the significant increases (concordant with increasing F^- dose) of F^- concentration observed in femora and vertebral bodies in all three strains.

This observation is important to note. It shows that there was a substantial accumulation of F^- in bone in all three strains. Furthermore it suggests that whatever the difference(s) that could be observed in F^- effects on bone between any of the three strains, they cannot be explained by a different concentration of F^- in bone.

Hypothesis 2: The bone density will be more affected in the susceptible strain.

Fluoride treatments did not affect femur and vertebral body bone mineral density (BMD) in the three strains, regardless of F^- dose (Figure 16). This observation is in contradiction with results from previous studies in which F^- treatment was shown to

increase the mineralization of bone in humans [220], rats [160], rabbits [164] and chickens [223]. This cannot be explained by low bone F^- concentrations as we demonstrated that there was a dose dependent increase of F^- in femora and vertebral bodies in all three strains. The fact that the mice were euthanized when they were still growing, even though at a lower rate, might play a role in this observation. The growth potential of these mice may mask the effect of fluoride on bone BMD.

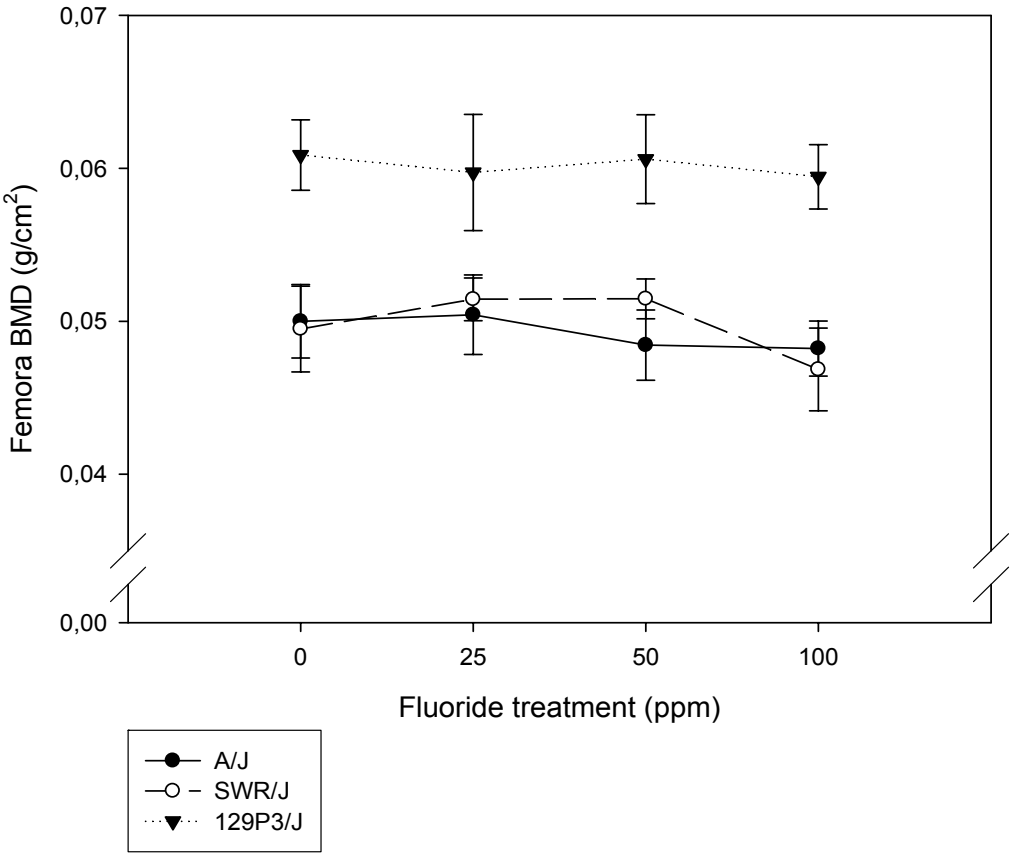

Figure 16: Femora BMD (g/cm^2) in each treatment group. Data are presented as means ± SD.

As already mentioned previously (see Introduction chapter), bone material is not isotropic and displays variations in tissue material properties, which are thought to be

associated mainly with local variation in degree of bone mineralization. This heterogeneity reflects bone turnover, mineralization kinetics and average bone matrix age [30]. This idea of heterogeneously mineralized bone has led to the concept of Bone Mineralization Density Distribution (BMDD) [87]. In contrast to BMD, which is an estimate of the total amount of mineral in a scanned area of whole bone, BMDD describes the local mineral content of the bone matrix throughout the sample. BMDD of healthy humans has been shown to have a remarkably small biological variance in adult trabecular bone [37].

In the present study, femur and vertebral body BMD was measured by DXA (Dual energy X-ray Absorptiometry). BMD is thus an areal measurement (areal BMD, g/cm^2) that cannot take into account changes in area versus volume in growing bones. Furthermore this measurement does not explore local variations in mineral contents and cannot be extrapolated to make any assumption about bone quality.

It must be noted that BMD has long been considered as the best parameter for predicting the risk of fracture in the elderly, as changes in BMD reflect changes in bone formation and/or bone mineralization. Although BMD has been extensively used as a predictor of bone fragility, it is becoming increasingly clear that it is not the sole determining factor [224]. Therefore, the lack of effect of F^- treatments on BMD in the three strains cannot predict a lack of effect of F^- on bone quality in the three strains.

Hypothesis 3: The mechanical properties of trabecular and cortical bone will be mainly altered in the susceptible strain.

Three-point bend testing of femora, compression testing of distal lumbar vertebral bodies and femoral neck-facture testing showed that F^- treatments affected differently bone mechanical properties in the three strains. Bone mechanical properties varied depending upon genetic background and F^- dose. With increasing F^- doses, alterations of bone mechanical properties were observed in A/J (susceptible) strain and SWR/J (intermediate) strain. No effect was detected in 129P3/J (resistant) strain. A/J strain showed significant alterations of bone mechanical properties in all three mechanical tests that are femur three-point bending tests (Figure 17), vertebral body compression tests and femoral neck-fracture tests. SWR/J strain showed a decrease of bone mechanical properties in vertebral body compression tests and femoral neck-fracture tests. As no change was discovered with F^- treatments in the gross morphology of femora and vertebral bodies in the three strains, the mechanical changes observed cannot be explained by alterations of the bone macroarchitecture. As bone F^- concentration was similar in the three strains, genetic variation in F^- uptake and excretion cannot explain the differences in mechanical properties observed between the three strains. Previous *in vitro* and *in vivo* studies have shown that bone mechanical properties are degraded in a dose-dependent fashion as F^- bone content increases [149, 172]. Nevertheless, to our knowledge, a genetic influence of F^- on bone mechanical properties in an *in vivo* mouse model has never been published.

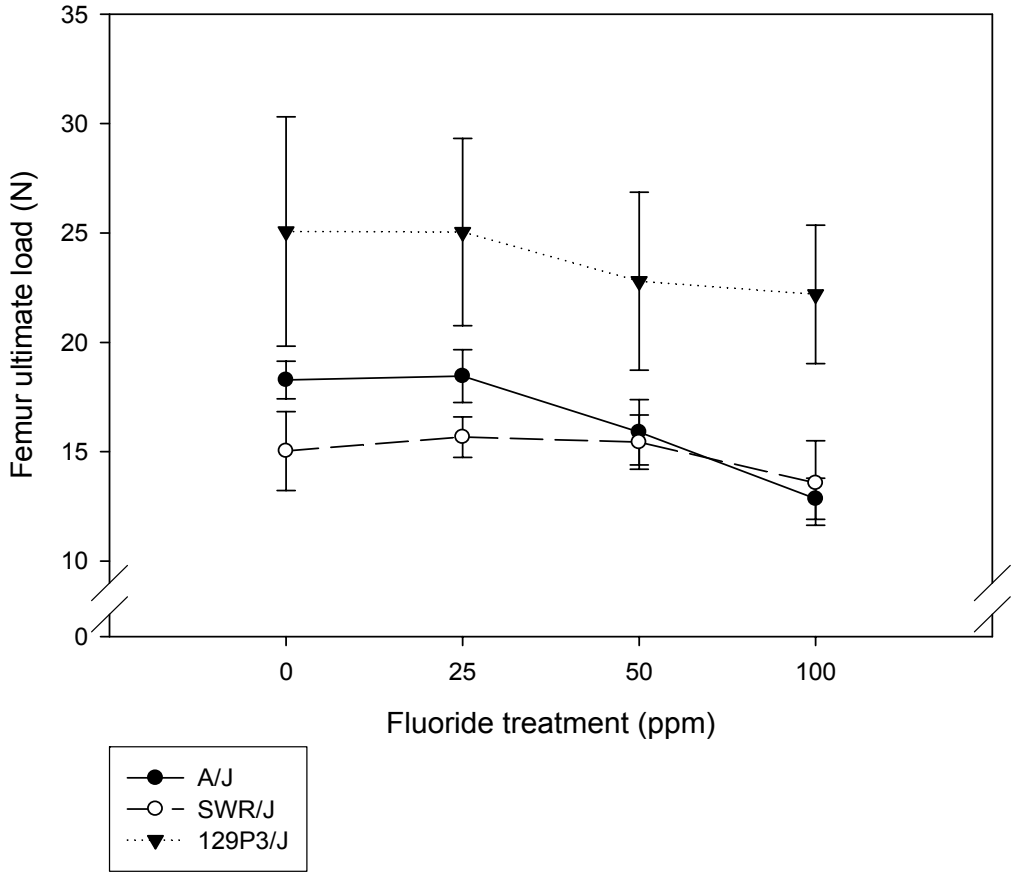

Figure 17: Femur ultimate load (N) in three-point bending test. Only A/J strain showed significant alterations of femur ultimate load. Data are presented as means ± SD.

Three-point bending results did not show any influence of the F^- treatments on bone mechanical properties in SWR/J strain although this strain showed significant mechanical alterations in the vertebral body compression test. This can be explained by the fact that genetic backgrounds may influence the mechanical behavior of cortical (femur) and trabecular (vertebra) bone differently. Indeed several studies have

demonstrated that cortical and trabecular bone may be differently regulated [100, 225, 226, 227].

Significant alterations in bone mechanical properties were found in both strains A/J and SWR/J although no change was noted in their femora and vertebral bodies BMD. This observation is in agreement with previous studies that showed that BMD measurements did not systematically correlate with the biomechanical properties of bone and therefore that change in BMD was not a good predictor of bone strength [162, 164, 168, 212] and of fracture risk [228].

It is interesting to note that at baseline 0ppm, comparison of bone mechanical properties between the three strains showed significant differences, with 129P3/J strain having "stronger" femora and vertebral bodies. Such differences between different strains of mice have already been described in the literature and can be explained by differences in bone macroarchitecture, bone microarchitecture and/or bone material intrinsic properties, which are influenced by genetic background [95, 96, 99, 100, 103, 225].

Hypothesis 4: Fluoride will induce bone histological and connectivity changes that will be more severe in the susceptible strain.

Strut analysis and microcomputed tomography of distal thoracic vertebral bodies showed that F^- treatments had no significant effect on trabecular bone microarchitecture in any of the strains. This is in agreement with previous studies showing that F^- therapy does not influence trabecular connectivity [229, 230]. Therefore the alterations in bone

mechanical properties observed for A/J and SWR/J strains with F^- treatment cannot be explained by altered bone microarchitecture.

The only static histomorphometric parameter that showed a significant change was the osteoid formation, with a significant increase in osteoid formation at the largest F^- dose in all three strains (Figure 18). High doses of F^- were shown to affect bone cells and therefore remodeling processes. Fluoride has been shown to have a mitogenic effect on osteoblasts [143, 155, 195]. Effects of F^- on osteoclasts have also been described [147]. The cumulative result of these effects is a net increase in bone formation. This is consistent with our observations of osteoid formation in all three mouse strains.

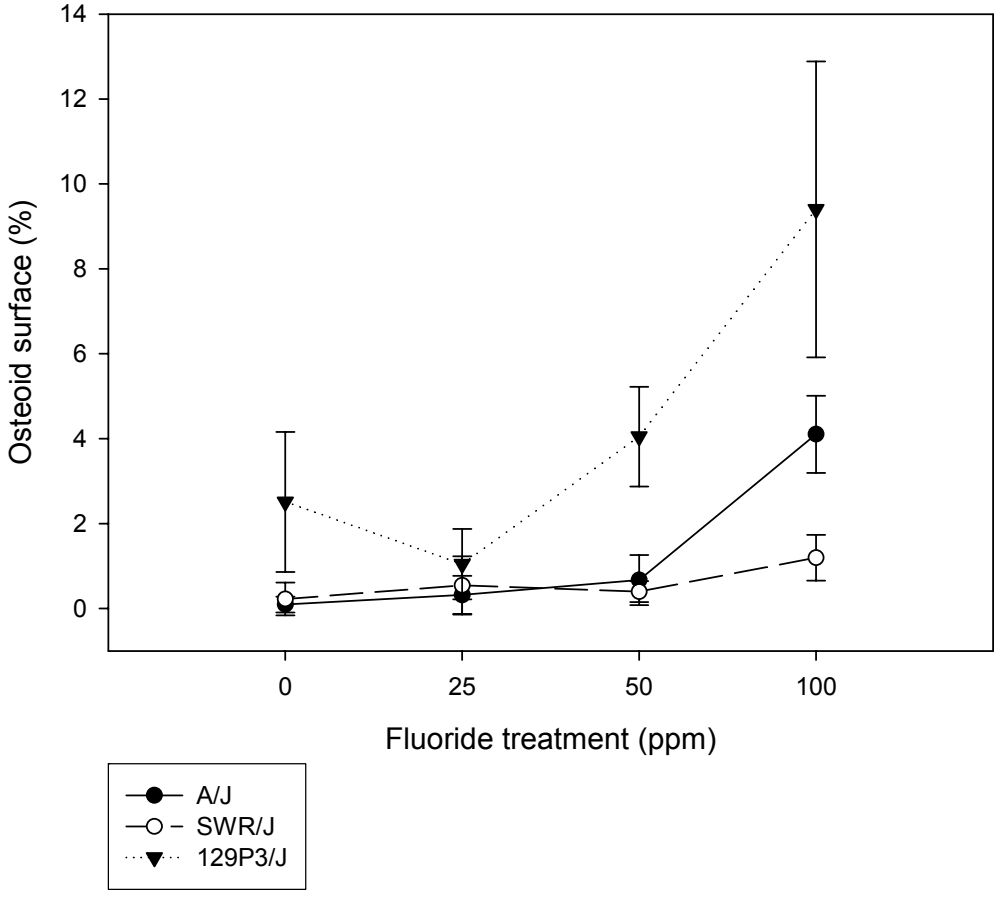

Figure 18: Osteoid surface in each treatment group.
A significant increase in osteoid surface was noted at the largest F^- dose in all three strains. Data are presented as means ± SD.

Although the overall osteoid formation was significantly larger in 129P3/J strain, the percent increase in osteoid volume, surface and thickness for the three strains correlated with their susceptibility to dental fluorosis. The percent increase in osteoid volume, surface and thickness was higher in the susceptible A/J strain, while the resistant 129P3/J strain demonstrated a less important percent increase in osteoid formation. Therefore it could be assumed that F^- effects on bone formation are influenced by genetics. Such an influence of genetics on F^- effects on bone cells has recently been described. Indeed F^- effects on osteoclastogenesis have been demonstrated to be influenced by genetic background [148].

Increase in trabecular thickness has been described with F^- treatments [229, 230]. Nevertheless, in our study, we did not find any change in trabecular thickness. The absence of significant changes may be explained by the fact that F^- was administered for only 42 days, which may be too short a period of time to have any significant effect on trabecular thickness.

Hypothesis 5: The accumulation of fluoride in bone will influence the mineralization and the material properties.

Fluoride has been shown to have an effect on bone mineralization. It retards mineralization and the mineral produced is less susceptible to dissolution [141, 153, 154]. Alterations in bone crystal structure have also been described [153]. In our study, the BSE mineralization profile of cortical and trabecular bone showed a non-significant increase towards higher mineralization with increasing F^- dose in the three strains (figure 19). This is consistent with the increased resistance to dissolution of bone

mineral with an increased F^- content. Fluoride treatment had different effects on mineralization heterogeneity of the trabecular and cortical bone. Vertebral body trabecular bone BSE imaging revealed significantly decreased mineralization heterogeneity in SWR/J strain at 50ppm and 100ppm F^-. This result is difficult to interpret but may be consistent with observations that F^- delays mineralization of new bone. The absence of an effect on cortical bone mineralization heterogeneity may be explained by site-specific differences in bone turnover, as there is a higher turnover in trabecular bone.

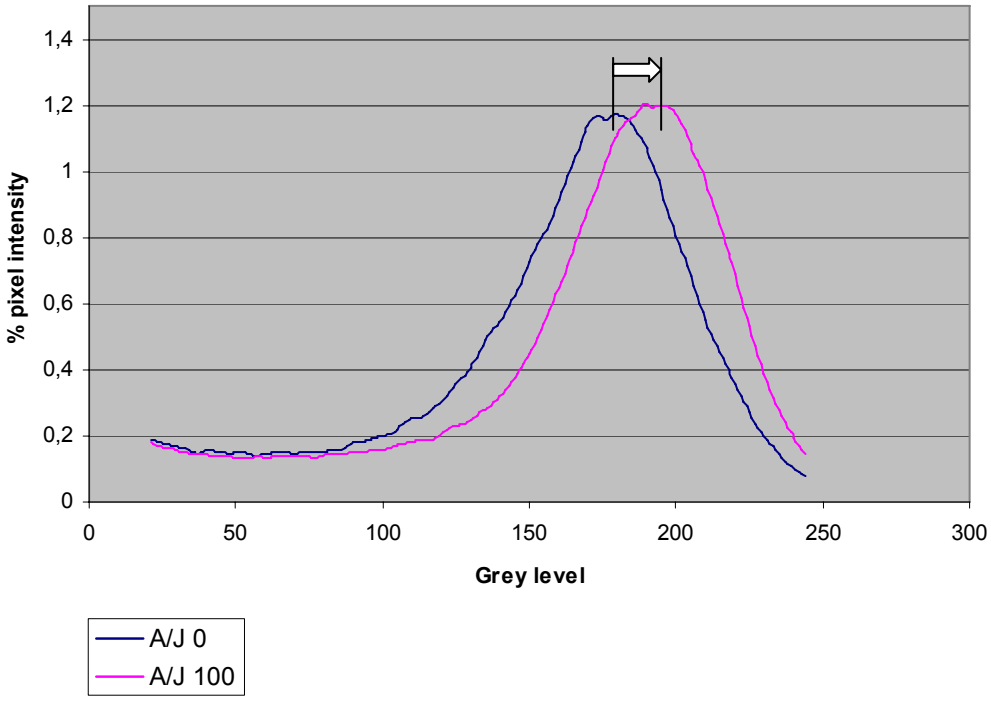

Figure 19: Evolution of the grey level distribution with fluoride treatment in the strain A/J for VB trabecular bone. The arrow indicates the right shift of the curve with fluoride treatment, meaning an increase of the grey level.

Powder x-ray diffraction study of femora showed significantly smaller crystals for 129P3/J strain, and increased crystal width with increasing F^- dose for all three strains (Figure 20). This relative increase in crystal width was similar in the three strains, but the absolute crystal size (length and width) remained smaller in 129P3/J strain, whatever the F^- dose. There was no effect of F^- on the crystal length in the three strains.

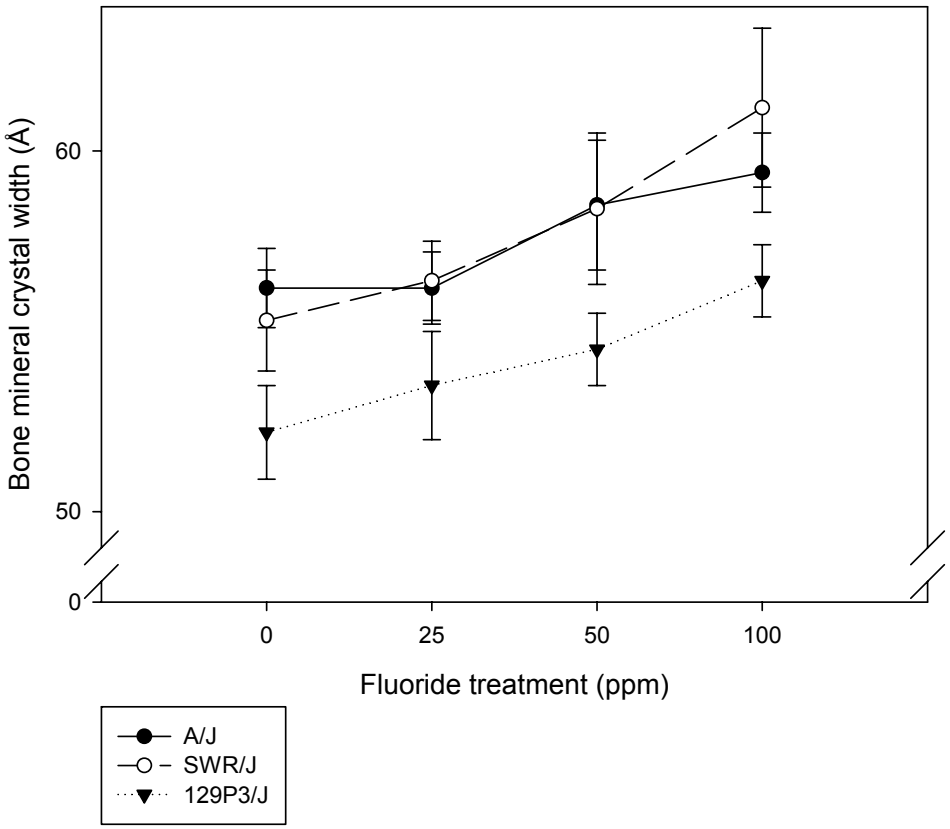

Figure 20: Crystallite width increase with fluoride treatment in the three strains. Data are presented as means ± SD.

In the literature, most authors accept that the crystal length is unchanged in F^- treatment [159, 160]; however there remains debate about changes in crystal width.

Some authors described an increase in crystal width [159, 161, 164], while others found no significant changes [160]. Eanes *et al.* [163] performed *in vitro* seeded growth experiments to determine whether this increase in apatite crystal width was physicochemical in origin (i.e., a direct result of F^- selectively enhancing lateral crystal growth), or an indirect consequence of F^--induced alterations in cellular function and matrix development. They found that F^- selectively stimulated HA crystal growth primarily by physicochemically interacting directly with the lateral faces of the growing bioapatite crystals and they suggested that F^- effects on bone cells played a secondary role in this process.

The overall smaller crystal size in 129P3/J strain suggests that the crystal growth rate of the resistant strain is slower than that of the other strains. This reduced crystal growth rate may be due to different mineral-collagen interface conditions that reduce the flux of calcium and phosphate ions to the apatite crystal surface. This observation suggests an influence of the genetic background on bone mineralization kinetics and on the quality of the mineral-organic interfacial bonding and/or bone matrix proteins.

The crystal width increase observed in the three strains with F^- treatment may be explained by an alteration of the mineral-organic interfacial bonding. *In vitro* studies have demonstrated such an effect of F^- on organic-inorganic interface [48, 165, 166]. Kindt *et al.* [166] studied the fracture surfaces of freshly fractured bovine and human bones that were exposed to highly concentrated sodium fluoride solutions. They showed an apparent loss of hydroxyapatite mineral crystals on a timescale of a few seconds, allowing the exposure of collagen fibrils situated beneath the crystals.

A correlation was found between the crystallite width increase and most of the observed decreased mechanical properties in the A/J and SWR/J strains (Figure 21).

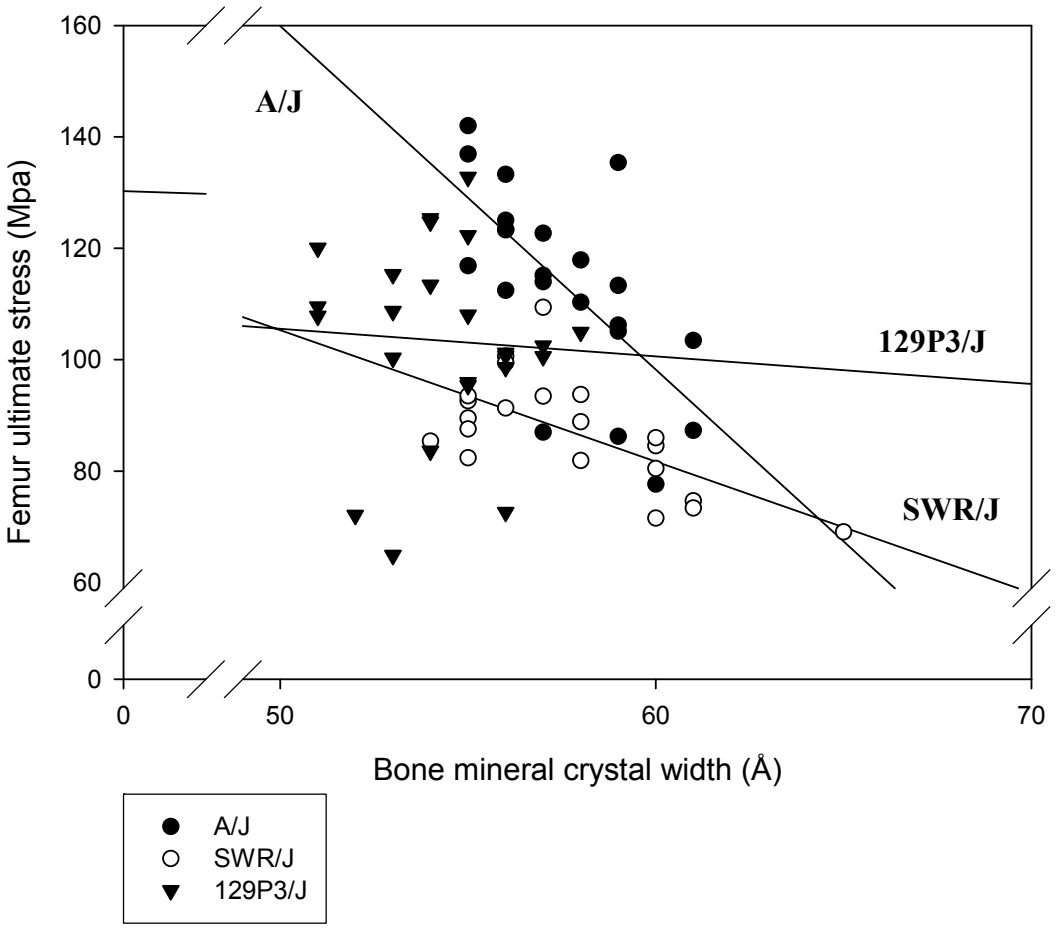

Figure 21: Correlation analysis between crystallite width and femur ultimate stress for each strain at all F^- dose treatments. Crystallite width increase correlates with the decreased femur ultimate stress observed in the susceptible (A/J) and intermediate (SWR/J) strains.

This observation is in agreement with the results obtained by Turner *et al.* [162] who showed a negative correlation between the increase of mineral crystal width and fracture stress of the femur in rabbits treated with F^-. These observations suggest that bone crystal size may play an important role in determining bone quality. The fact that the 129P3/J resistant strain has the strongest bone and the smallest bone crystal may suggest that these mice have a stronger mineral-organic interfacial bonding, which has previously been shown to play an important role in bone quality [48].

To test bone mechanical properties at the microscopic level, a microhardness study was performed on trabecular bone (thoracic vertebral body) and cortical bone (thoracic vertebral body and distal femur). This test has the theoretical advantage of eliminating architectural considerations, instead focusing on the tissue-level mechanical properties of the bone. The hardness of bone is linked to its degree of mineralization and should be proportional to bone stiffness. In the present study, there was no effect of F^- on trabecular and cortical bone microhardness. This is in contradiction with other studies that described a decreased bone microhardness [219] or an increase of the microhardness of both cortical and trabecular bone with F^- treatment [164]. This discordance may be explained by the lack of precision of this test. Variations in trabecular shape, orientation and number, as well as variations in mineral distribution and properties throughout individual trabeculae, are known to influence the results of this test [85, 231, 232]. This lack of precision is also present in cortical bone microhardness study, as we tested femur and VB cortical bone microhardness at one cross-section only. The absence of any change in microhardness may also be explained by the short period of F^- treatment and by the fact that F^- was given to rapidly growing mice. Therefore we cannot draw any certain conclusions from the absence of significant differences in the results.

4.2. Model for Fluoride Action on Bone Material Strength

The results obtained in the present study allow us to propose a model that could explain the effect of mineral-collagen interface quality and F^- concentration on crystal growth and composite material strength (Figure 22).

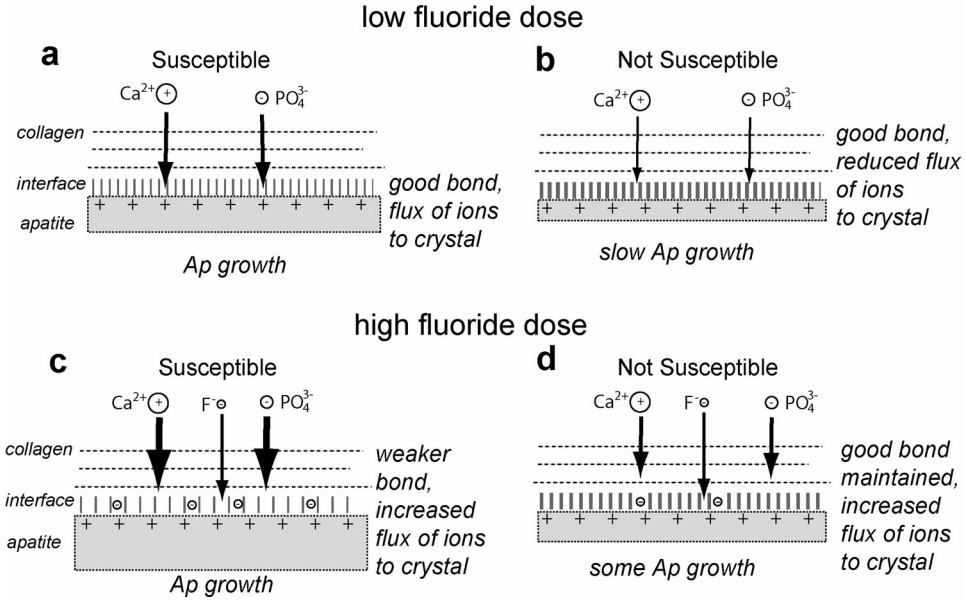

Figure 22: Schematic of the effect of mineral-collagen interface quality and fluoride concentration on crystal growth and composite material strength.

At low F^- dose (Figure 22, a and b), the 129P3/J resistant strain has a stronger organic-inorganic interface than the strains A/J and SWR/J. This different organic-inorganic interface may be due to differences in matrix proteins that bind to the bone crystals. Indeed it has been proposed that extracellular matrix proteins that bind with high

specificity and avidity to the crystal nucleus, stabilizing the nucleus and facilitating its growth, can, in higher concentrations, bind to similar sites and block crystal growth [39, 233]. This strong interfacial bonding may reduce crystallite growth rate, explaining the smaller bone crystals discovered in the 129P3/J strain, and may increase the composite material strength. *In vitro* studies have demonstrated that F^- ions could alter bone mechanical properties by altering mineral-organic interfacial bonding [48, 165, 166]. At high F^- dose (Figure 22, c and d), such interfacial bonding alteration could weaken the mineral-organic bond and reduce interfacial mechanical strength. Theoretically the exchange of F^- for hydroxyl ions should not alter the electrostatic equilibrium since both have the same valence (-1), but F^- ions cannot form hydrogen bonds as can hydroxyl ions. Another mechanism that may be involved in the alteration of interfacial bonding by F^- is a local increase in pH which may alter protein secondary and tertiary structures [88]. The weaker mineral-organic interface allows an increased flux of calcium and phosphate ions to the apatite crystal surface, which explains the increased crystal width observed in the three strains. This increase in crystal width was similar in the three strains and the resistant 129P3/J strain had still the smallest bone crystals at high F^- dose, which may explain the absence of bone mechanical properties alteration in this strain.

4.3. Critics

A multitude of extrinsic and intrinsic factors are known to influence the size and composition of bone mineral crystals [22]. Among the intrinsic factors, the properties of the collagen and the distribution of other matrix protein that are involved in nucleation and growth of the mineral crystals are important factors that were not specifically studied in the current study. Therefore another possible mechanism of F^- action could be an alteration of bone matrix proteins, i.e. collagen and noncollagenous proteins.

Collagen and noncollagenous proteins are of significant importance for the biomechanical integrity of the bone [76, 79, 80, 232] and many bone matrix proteins play important roles in mineralization [15]. Miao *et al.* [145] have investigated the effects of F^- treatment (221 mg/L NaF in drinking water for two months) on rat calvarial osteoblasts. They showed that excessive F^- intake could inhibit the synthesis of type I collagen and decrease the degree of collagen cross-linking. An influence of F^- on proteoglycan structure synthesized by mineralizing bone cells [234] and on expression of matrix metalloproteinases [235] has also been proposed. Waddington *et al.* [234] demonstrated an influence of F^- on proteoglycan structure synthesized by mineralizing bone cells, using a bone mineralizing culture system derived from rat femur washes. They suggested that F^- could affect the post-translational assembly of the GAG chains which may influence the mineralization process. They also showed an altered expression of some matrix metalloproteinases in the presence of F^-, using the same *in vitro* culture model [235]. This influence of F^- on metalloproteinases expression was proposed to influence the composition of the remodeling matrix and subsequent mineralization. Kindt *et al.* [166] showed that enhanced formation of superficial fluoroapatite weakened the protein-hydroxyapatite interfaces but NaF was also found to

be a potent agent for extracting noncollagenous proteins from bone powder. It must be pointed out that these effects were demonstrated in *in vitro* studies and that they may be different *in vivo*.

Therefore to be complete, the present study should also look for any alteration in bone matrix proteins, in order to assess the contribution of the genetic background in the changes observed in the quality of new bone (resulting from the anabolic action of F^-).

4.4. Conclusions

Although fluoride therapy has been used clinically for many years as a treatment for osteoporosis, its use was controversial with many unanswered questions regarding its mechanisms of action and its real effect on bone strength. In the literature we can find many animal and clinical studies that describe controversial results about the effect of F^- on bone mechanical properties, some studies describing an adverse effect of F^- on bone properties [162, 168, 171, 236] while a few reported an increased bone strength [193]. Although an influence of the genetic background has long been suggested by epidemiological studies [131, 204, 205, 206, 207], no animal model has been developed to clearly study this influence of genetics on F^- action on bone properties.

Fluoride is known to have biological effects on bone cells, as well as chemical and physical effects on bone crystals. In the present study, increasing F^- doses had strain-specific effects in mice. Mechanical testing showed significant alterations in bone mechanical properties in the susceptible A/J strain, whereas moderate alterations in the intermediate SWR/J strain and no effects in the 129P3/J strain were observed. Fluoride

treatment had no effect on BMD as well as bone macro- and micro-architecture in the three strains. The increased osteoid formation and decreased mineralization heterogeneity support the theories that F^- stimulates osteoblastic activity and delays mineralization of new bone. The 129P3/J resistant strain was shown to have smaller bone crystals and a correlation was found between the F^- induced increase in crystal width and most of the decreased mechanical properties in both A/J and SWR/J strains.

Based on these results, we can postulate that the adverse effect of F^- on bone mechanical properties may be explained by different mechanisms that are linked and are in relation with bone mineralization:

- Fluoride induces an increase in bone crystal width which may play an important role in determining bone strength. If there is an increase in crystal width with F^- treatment, it may be postulated that the specific surface of the HA crystal on which the collagen can bind could become smaller [153, 158], which may be responsible of an alteration in bone mechanical strength.
- This crystal width increase may suggest a negative effect of F^- on the quality of the mineral-organic interfacial bonding and/or bone matrix proteins which are known to play an important role in bone quality [48, 165].
- As crystal width is increased, it has been proposed that crystals of this size could hardly be located inside the collagen fibrils and that F^- lead to an enhanced extrafibrillar mineralization [218]. This location of additional large crystals outside the collagen fibrils may contribute to the decreased bone mechanical properties.

The present study emphasizes the importance of considering bone mineral crystal size, mineral-organic interfacial bonding and bone matrix proteins in understanding bone quality. It also emphasizes the importance of considering genetic background when studying bone quality. Not only are bone properties influenced by genetics, but genetics

also play an important role in the interaction between extrinsic factors and bone metabolism. In the present study, F^- action on osteoblasts activity was clearly influenced by genetics as we showed an increase in osteoid formation, which correlated with strain's susceptibility to dental fluorosis.

An influence of genetics on bone mineralization (mineral content, mineral crystallinity) has already been described [22]. In the present study, the resistant strain had initially (at 0ppm F^- treatment) smaller mineral crystals compared to both other strains. Fluoride treatment induced an increase in crystal width with increasing F^- dose in all three strains. It is interesting to note that this relative increase in crystal width was similar in the three strains, explaining that the absolute crystal size (length and width) remained smaller in the resistant strain, whatever the F^- dose. Therefore it seems that the influence of the genetic background on F^- effect on bone crystal size is not direct but depends on the organic-inorganic interface quality of the strain, which is influenced by genetics.

In conclusion, even though individuals receive small amounts of F^-, this ion accumulates passively in bone mineral over a time span of decades. This situation is difficult to model in animals and therefore remains poorly understood. The possible impact of the ingestion of small amounts of fluoride, over a life time, on the skeleton depends on each individual's genetic background.

4.5. Applications and Future

Fluoride is known to alter mineralization within bone, although the mechanism for its action is still unclear. The present study suggests an action of F^- on bone mineral crystals via an alteration of mineral-organic interfacial bonding and/or bone matrix proteins, emphasizing the importance of these parameters in bone quality. It also shows the marked influence of the genetic background on the quality of this mineral-organic interfacial bonding and/or these bone matrix proteins. Nowadays we are lacking techniques allowing a detailed study of this interface and most of our knowledge is based on *in vitro* experiments. As the mineral-organic interface quality is thought to have an important influence on the mechanical behavior of bone, it would be interesting to quantify the "mineral-organic bonding effectiveness". Bundy [237] has proposed a theoretical mathematical model to determine the effectiveness of the mineral-organic bonding as well as the amount of debonding that could be found in some pathological situations like osteoporosis. Nevertheless, this paper gives only theoretical considerations and a technique allowing the quantification of the interface quality has still to be developed. As interface quality may be by itself an independent risk factor for fracture, such study would also improve the assessment of the fracture risk, which is of particular clinical interest for osteoporosis treatment. Nowadays, diagnosis and management of patients who may be at risk for or who have suffered osteoporosis-related fractures are still a challenge and a better definition of the fracture risk may help not only with detection of patients at risk but also with assessment of treatment effects.

Genetic factors play a major role in the determination of osteoporosis risk. More and more genetic studies have clearly identified numerous genes associated with BMD and/or fragility fractures [238]. Nevertheless most genetic risk factors have still to be

discovered. Genetic study of the inbred strains of mice used in the present study could allow the identification of chromosomal loci and genes associated with quality in mineral-organic interfacial bonding and/or bone matrix proteins. Finding genes that predict bone quality and thus the risk of fractures in humans will remain a challenging endeavour for the foreseeable future.

It is increasingly accepted that interactions with environmental factors must play an important role in determining bone quality and that these interactions must be influenced by genetics [116, 239]. There are numerous environmental factors that may modulate expression of one or more genes [240]. The present study shows the important influence of the genetic background on the action of F^- on bone quality. Understanding gene-environment interactions might allow us to give individualized preventive advice before disease diagnosis, in addition to offering personalized treatment after a disease, or a disease susceptibility, has been diagnosed.

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ABSTRACT

Nowadays it is increasingly accepted that interactions with environmental factors must play an important role in determining bone quality and that these interactions must be influenced by genetics. Nevertheless, to date, only few animal studies explore an underlying genetic basis for extrinsic factors effect such as fluoride (F^-) effect on bone metabolism.

This study assessed the effect of increasing F^- doses on the bone properties in three inbred mouse strains that demonstrate different susceptibilities to developing enamel fluorosis (A/J a "susceptible" strain, 129P3/J a "resistant" strain and SWR/J an "intermediate" strain). Fluoride concentrations were determined in femora and vertebral bodies. Bone mineral density was evaluating through DXA (Dual energy X-ray Absorptiometry). Three-point bend testing of femora, compression testing of vertebral bodies and femoral neck-fracture testing were performed to evaluate mechanical properties. Bone microarchitecture was quantified with microcomputed tomography and strut analysis. Bone formation was evaluated by static histomorphometry. Bone mineralization was quantified with backscattered electron (BSE) imaging and powder x-ray diffraction. Microhardness measurements were taken from the vertebral bodies (cortical and trabecular bone) and the cortex of the distal femur.

Increasing doses of F^- had different effects on the bone properties in the three inbred mouse strains. Although significant increases of bone F^- concentration could be demonstrated, and no significant effects on bone macroarchitecture and BMD were found in the three strains, mechanical testing showed significant degradation of bone mechanical properties in the "susceptible" A/J strain, whereas moderate degradation in the "intermediate" SWR/J strain and no effect in the "resistant" 129P3/J strain were observed. Evaluation of the structural and material properties of the bone in the three strains showed that this different effect of F^- on bone mechanical properties could be explained by an alteration of the mineral-organic interfacial bonding and/or bone matrix proteins, interfering with bone crystal growth inhibition on the crystallite faces as well as bonding between the mineral and organic interface. The smaller bone crystallites of the 129P3/J (resistant) strain may indicate a stronger organic-inorganic interface, reducing crystallite growth rate and increasing interfacial mechanical strength.