Bone: functions, structure and physiology

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Abstract In this chapter, bone functions, regulation, morphological structure and physiology are revisited. Bone is a highly complex tissue, very sensitive and responsive to external and internal stimuli, and intimately intertwined with other organs. From embryogenesis to endocrine regulation and bone remodelling, a global assessment is presented. Considering the scope of this book, special emphasis is given to how cell structure and tissue organization modulate the response to mechanical stimuli.

1. Introduction

The deeply dynamic nature of bone may be missed by a less attentive eye. Bones are resilient, and apparently quite rigid structures. They vary in shape, size and number, and are divided in the axial and appendicular skeleton. Through life, they are subjected to loads and strains that temper their shape, with old matrices being replaced by newly formed ones, maintaining bone volume and strength. When trauma and fractures occur, bones are capable of healing if enough stability and proper alignment are guaranteed.

Osteogenesis, bone repair and remodelling are directed by the exchanges involving the environment, cell-to-cell interactions and cell-extracellular matrix.

Mechanical forces are crucial in early embryonic development. Morphogenesis is controlled through fluid flow mechanisms and by cellular contractility. Early embryo shaping depends on morula contraction, determined by cohesivity. Multiple

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layers result, with the development of endoderm, mesoderm and ectoderm in the blastula (Oster et al., 1983; Takeichi, 1988; de Vries et al., 2004; Ingber, 2006). Mechanical forces, cell geometry, and oriented cell division together orchestrate normal airway tube morphogenesis (Tang et al., 2018) and may help determine the neocortical organization (Foubet et al., 2018). Cells generate tension through contraction of actin-myosin cytoskeleton filaments, which are transmitted through cadherin-mediated adhesion sites to surrounding structures, these being either cells or extracellular matrix. The cytoskeletal conformation and cell shape stress-dependent changes regulate cell phenotype; interactions with the extracellular matrix are of paramount importance for cell phenotype (Ingber, 2006). Organ lateralization and asymmetry depend on unidirectional fluid flow, generated by specialized motor protein complex dynein. The fluid flow induces differences in key molecules expression (such as the TGF-family signalling molecules) (Collignon et al., 1996, Okada et al., 1999; Cartwright et al., 2004, Nakamura et al., 2006). Lateralization may also depend on fluid shear, in the embryo, by acting on a group of non-motile cilia, coupled to calcium channels; fluid flow generated shear may cause the intracellular calcium concentrations to increase and initiate the cascade of events responsible for lateralization (McGrath et al., 2003).

The mechanical environment is also a determining factor for vasculogenesis, angiogenesis (Schmidt et al., 2007; Patwari and Lee, 2008), and neuronal development (Bray, 1979; Dennerll et al., 1989; le Noble et al., 2008; Anava et al., 2009).

The embryo mesoderm is constituted by spindle or star-shaped cells called mesenchymal stem cells (MSCs). MSCs are the utmost pluripotential cells in the organism, originating different tissues such as the connective tissue, muscle, cardiovascular tissue and the whole skeletal system. Bone, cartilage, tendons and ligaments develop through mechanisms of proliferation, migration and differentiation, but also programmed cell death/ apoptosis (Carter and Beaupré, 2001).

We now beginning to address how complex bone is in its functions, how its macro-architecture, microarchitecture and arrangement at molecular level play together remarkably, ensuring its responsiveness to external and internal stimuli and close entwining with other organs.

2. The complexity behind simplicity

2.1 Bone functions

Osseous tissue is the most rigid and resilient tissue of the body. Bone is composed of dense connective tissue, it is the primary skeleton component, thus providing structure, support, and protection to vital organs, like the brain (skull), the spinal cord (vertebrae), and the heart and lungs (ribs and sternum). Vertebrae also participate in the spine shock absorbance – providing adequate load cushioning for

the fibrocartilaginous joints at the intervertebral disks. Long bones provide structure, stability and, along with the joints, enable body movement – providing levers for the muscles.

Moreover, bones act as the major source of blood, since haematopoiesis occurs in their medullary cavity. In infants, the bone marrow of all long bones is capable of blood synthesis. With ageing, part of the red marrow turns into yellow fatty marrow, no longer capable of haematopoiesis. Functional red marrow in adults is limited to the vertebrae and the extremities of femur and tibia.

Bones also partake a vital role as:

- Mineral storage: mostly calcium, phosphate, and magnesium; bone plays an important metabolic role, mediated by several hormones, regulating mineral homeostasis (Bélanger et al., 1969; Zallone et al., 1983; Teti & Zallone, 2009).
- Acid-base balance, as bone can buffer blood against extreme pH changes by absorbing or releasing alkaline salts, through bone cells activity (Green & Kleeman, 1991; Arnett et al., 2003; Bushinsky & Krieger, 2015).
- Osteoblasts have been shown to produce growth factors, with production regulated by systemic hormones and local mechanical stress (Baylink et al., 1993). The bone matrix holds several growth factors such as insulin-like growth factors I and II, transforming growth factor-beta, acidic and basic fibroblast growth factor, platelet-derived growth factor, and bone morphogenetic proteins, released when resorption occurs (Linkhart et al., 1996).
- Adipose tissue storage (yellow bone marrow functions as a fatty acid/ energy reserve) (Rosen et al., 2009; Krings et al., 2012; Suchacki et al., 2016).
- Heavy metals and other foreign elements, after detoxification from the blood, are stored in bone and later excreted (Roelofs-Iverson et al., 1984; Sharma et al., 2014).
- Bone functions as an endocrine organ, as it produces two known circulating hormones:
    a. Fibroblast Growth Factor 23 (FGF23): FGF23 was first described by Yamashita et al. and it is produced mainly by osteocytes (Yamashita et al., 2000; Rhee et al., 2011), but also by osteoblasts (Masuyama et al., 2006). FG23 acts on the kidneys, inhibiting 1α-hydroxylation of vitamin D and promoting phosphorus excretion in urine (Shimada et al., 2004; Fukumoto & Martin, 2009; Haussler et al., 2012). FGF23 also decreases phosphorus absorption in the intestine, regulating inorganic phosphate metabolism and thus, mineralization (Fukumoto & Martin, 2009). Serum calcium concentration regulates FGF23 production (David et al., 2013), thus making FGF23 into a calcium-phosphorus regulatory hormone (Rodriguez-Ortiz et al., 2012).

Hence, FGF23 excess or deficiency results in anomalies of phosphate metabolism. Excess FGF23 hinders renal phosphate reabsorption and 1,25 dihydroxy vitamin D_3 [1,25$(OH)_2$D] production, causing hypophosphatemia and suppression of circulating 1,25$(OH)_2$D levels and, eventually, rachitic changes in bone (Fukumoto & Yamashita, 2007). These changes occur in autosomal dominant hypophosphatemic rickets and osteomalacia (ADHR Consortium, 2000) and in tumour-induced osteomalacia (TIO), a paraneoplastic syndrome (Shimada et al., 2001). In contrast, reductions in FGF23 cause tumoral calcinosis syndrome, characterized by hyperphosphatemia, increased 1,25$(OH)_2$D and soft tissue calcifications (Lyles et al., 1988; Fukumoto & Yamashita, 2007). An obligate FGF23 coreceptor was identified – Klotho (Urakawa et al., 2006). Klotho is essential to activate FGF receptors and their signalling molecules. Secreted Klotho suppresses either by direct interaction or interference with receptors, the activity of several growth factors: insulin, insulin-like growth factor-1 (IGF-1) (Kurosu et al., 2005), Wnt (Liu et al., 2007), and TGF-β1 (Doi et al., 2011). The FGF23-Klotho axis represents a specialized system responsible for the external and internal calcium and phosphorus balance in the bone, intestine and kidney. FGF23-Klotho axis works under parathormone regulation, with parathormone increasing serum FGF23 levels and directly promoting FGF23 expression by osteocytes (Lopez et al., 2011; Quarles et al., 2012); FGF23 exerts negative feedback by inhibiting the parathyroid glands (Ben-Dov et al., 2007; Krajisnik et al., 2007). FGF23 production in the osteocyte may be inhibited by osteopontin (Paloian et al., 2016).

b. Osteocalcin is a protein produced by osteoblasts in bone, and it is a major regulator of insulin secretion by direct action over the pancreatic β-cell. Osteocalcin also increases insulin sensitivity of peripheral tissues, e.g. muscles and liver, up-regulating glucose uptake and energy expenditure, thus contributing to glycaemia regulation (Lee & Karsenty, 2008; Ferron et al., 2008; Ferron et al., 2010; Fulzele et al., 2010); it also reduces fat deposition by inducing adiponectin secretion by adipocytes (Ribot et al., 1987; Reid et al., 1992). Blood osteocalcin levels are significantly lower in diabetic patients when compared to non-diabetic controls, and osteocalcin levels are inversely related to fat mass and blood glucose (Kindblom et al., 2009; Pittas et al., 2009). Lastly, osteocalcin influences male fertility, by enhancing testosterone production by Leydig cells in the testes (Oury et al., 2011).

2.2 Bone structure and mechanical behaviour

Bone is a composite material; the inorganic portion of bone comprising 70% (of which 95% is hydroxyapatite and 5% are impurities impregnated in

hydroxyapatite), whilst 22% to 25% are organic (of which 94-98% are mainly collagen type I and other non-collagen proteins and 2%-5% are cells); 5 to 8% is water (Sommerfeldt & Rubin, 2001).

Bone mechanical properties depend on porosity, composition, mineralization degree and organization of solid matrix. Therefore, the mechanical behaviour of an entire bone is highly dependent on its properties at a microscale (Rho et al., 1998; Augat & Schorlemmer, 2006).

Bone can be classified accordingly to its structural features at a microscopic level in woven and lamellar bone.

Woven bone is immature or pathologic, primary bone and it is present in growth, fracture healing and diseases such as Paget's disease. Cells and matrix are laid randomly. Woven bone is formed during intramembranous, endochondral or rapid appositional bone growth. In large animals (whether reptiles, birds or mammals), woven bone with large vascular canals is rapidly deposited in the subperiosteal region. Canals are lined with osteoblasts that gradually deposit lamellae until the canal has a reduced diameter; the resulting structure is a primary Haversian system or osteon. The random distribution of its components explains woven bone's isotropy.

Lamellar bone is organized, mature bone, morphologically classified into two different types: cortical or compact and cancellous or trabecular bone. Cortical and cancellous bone types differ in both structure and functional properties but both are highly anisotropic.

The typical structure of a long bone, such as the femur or the humerus, comprises the cylindric shaft, or diaphysis, and the extremities, or epiphyses (Fig. 1). The outer surface is covered by a layer of dense connective tissue called periosteum, except for the areas of mobile articulation, covered with hyaline cartilage. The periosteum is highly vascularized and responsible for appositional bone growth. The endosteum is a thin layer of connective tissue that lines the inner surface of the diaphysis, containing the medullary canal. The epiphysis consists of an outer layer of cortical bone surrounding the porous network formed by trabecular bone. Within the spaces between trabeculae lays red bone marrow (Van De Graaff, 2001). Long bones, fundamental for load bearing and leverage, evolved as structures in which stiffness along the long axis was favoured.

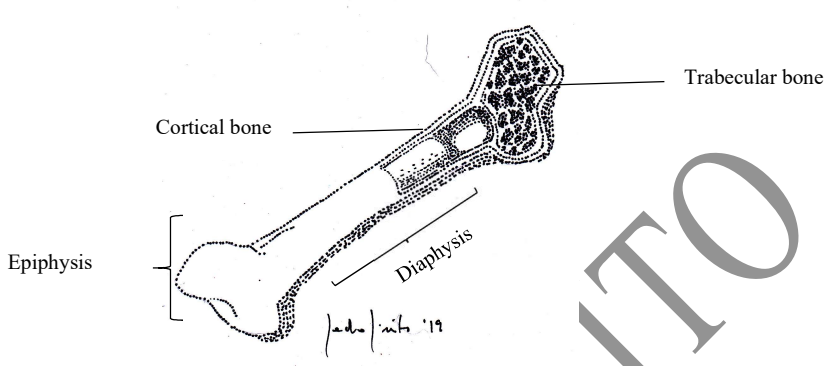

Fig. 1 Illustration of a long bone structure, showing the distribution of two different types of lamellar bone: cancellous and cortical compact bone.

Cortical bone (Fig. 2) accounts for approximately 80% of the skeletal mass. Cortical bone is vital to skeletal mechanical competence, both of long and flat bones. It is formed by tightly aligned collagen fibrils, making concentric lamellae. Each lamella is 2 – 3 μm thick and is arranged in distinct layers of parallel fibrils, each layer with a different fibril orientation (Weiner et al., 1999). Mineralization occurs by apatite crystals (mainly carbonated apatite) deposition within and around these fibrils. The lamellae form cylinders containing a hollow central canal where blood vessels and nerves run, composing the cortical bone microstructural unit, called Haversian system or osteon. From the centre of the osteon (Haversian canals), blood vessels form a three-dimensional network and penetrate the cortical bone layer perpendicularly (running within Volkman's channels) (Meyer & Wiesmann, 2006). In between the osteons are incomplete osteons, known as interstitial systems or interstitial bone.

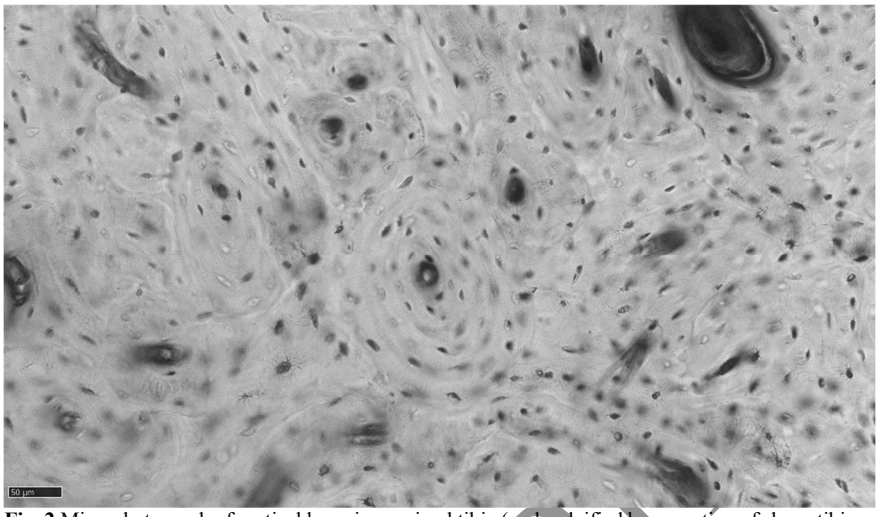

Fig. 2 Microphotograph of cortical bone in proximal tibia (undecalcified bone section of sheep tibia, Giemsa-Eosin, 40x magnification; slide digitalized using Nanozoomer SQ, Hamamatsu Photonics, Portugal). Haversian systems are evident, as are the concentric lamellae. Osteocytes are visible in their lacunae, in between lamellae.

Cancellous (or trabecular) bone is highly porous and adapted to compressive loads. The lamellae are organized in a parallel manner, forming trabeculae. These rod- and plate-shaped struts are organized into a flexible lattice with variable degrees of interconnectivity (Fig. 3).

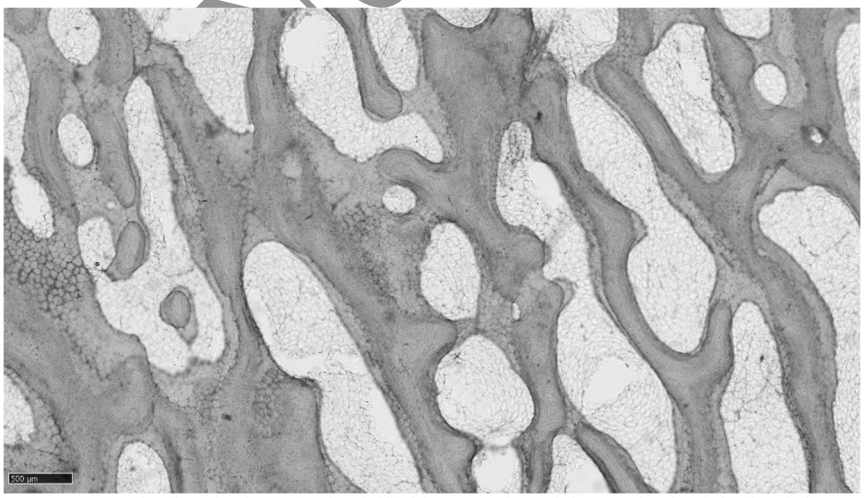

Fig. 3 Image of trabecular bone (undecalcified bone section of the proximal epiphysis of sheep's tibia, Giemsa-Eosin, 5X magnification; slide digitalized using Nanozoomer SQ, Hamamatsu Photonics, Portugal). The picture illustrates the sponge-like structure of cancellous bone.

The trabecular network is light and of utmost importance for load transfer in long bones, absorbing and distributing sudden stresses. In vertebrae, cancellous bone is the main load-bearing structure and essential for shock absorption. Trabeculae are approximately 200 μm thick and are orientated according to routine load bearing direction (Oftadeh et al., 2015). This is evident in epiphyses and metaphyses of long bones, but also in the vertebrae and ribs. Trabeculae are covered by osteoblasts and bone-lining cells. Osteoblasts actively lay extracellular matrix (ECM) and bone-lining cells are in an inactive state. The metabolic rate of trabecular bone is higher than that of cortical bone and the remodelling phenomena more prominent. (Carter & Beaupré, 2001; Currey, 2003).

Bone endures both compressive and tensile stresses. Bone is subjected to bending and torsion (Sommerfeldt & Rubin, 2001). In humans, there is a large variation in strains, ranging from to 400 to 2000 μstrains or even as high as 4000 μstrains (Duncan & Turner, 1995; Burr et al., 1996; Sommerfeldt & Rubin, 2001).

The bone exhibits a stress-strain response of sequential elastic and plastic responses. In its elastic region, no permanent damage is caused to the bone structure; if the stress increases, a gradual transition to a plastic response occurs. Post-yield deformations are permanent and cause trabecular fracture, cement lines and cracks. Crack formation and growth allow energy dissipation and are a powerful stimulus for bone remodelling in healthy bone.

The mineral component contributes to compression strength, while collagen fibrils are fundamental for tensile strength. The mineral phase is highly related to stiffness, whilst collagen is determinant for toughness (Zioupos et al., 1999). A higher Young's modulus corresponds to less ductility and higher brittleness (Turner, 2006).

Bone material properties reflect, therefore, high functional specialization and depend on architecture, composition and component spatial distribution.

2.2.1 The bone matrix

Structure and material properties of the bone depend on collagen. The collagen I molecule is composed of three long peptide sequences, arranged helicoidally. Collagen is produced by osteoblasts and goes through several enzymatic modifications whilst still within the cell (Young, 2003). After leaving the cell, collagen molecule undergoes further cross-linking within itself and with other collagen molecules. Collagen chain mutations lead to diseases such as osteogenesis imperfecta (Young, 2003; Bodian et al., 2009). The triple tropocollagen units are aligned in fibrils and display a permanent dipole moment. Consequently, collagen acts as a piezoelectric and pyroelectric material, and as an electromechanical transducer (Fukada & Yasuda, 1964; Noris-Suárez et al., 2007). The native polarity and the piezoelectric properties of collagen are associated with the mineralization process. Under compression, negative charges on the collagen surface become

uncovered and attract calcium cations, which are then tailed by phosphate anions (Noris-Suárez et al., 2007; Ferreira et al., 2009). Collagen can actively control mineralization, functioning in synergy with other non-collagenous proteins, inhibitors of hydroxyapatite nucleation. The positive net charge close to the C-terminal end of the collagen molecules promotes the infiltration of the fibrils with amorphous calcium phosphate; at the gap and overlap regions of the collagen molecule, the clusters of charged amino acids form nucleation sites and the amorphous calcium phosphate is changed into parallel oriented apatite crystals (Nudelman et al., 2010).

Non-collagenous proteins such as osteopontin, fibronectin, osteonectin and bone sialoprotein are present in much smaller quantities but are, nonetheless, essential for normal bone function and properties.

Osteopontin (OPN) is a non-collagenous glycoprotein, present in the bone matrix, binding to the cell surface and hydroxyapatite. It is mostly produced by proliferating pre-osteoblasts, osteoblasts and osteocytes, but also by fibroblasts, osteoclasts and macrophages (Ashizawa et al., 1996; Perrien et al., 2002). OPN intervenes in cell migration, adhesion, and survival in diverse cell types. OPN is a key player in bone remodelling processes. Its production is modulated by mechanical loading, being up-regulated both by loading and by loading deprivation (Harter et al., 1995; Perrien et al., 2002; Gross et al., 2005). OPN has been proved to inhibit mineralization but its deficiency significantly lessens bone fracture toughness and causes anomalous mineral distribution, leading to increased FGF23 production (Fisher et al., 2001; Jahnen-Dechent et al., 2008; Thurner et al., 2010, Paloian et al., 2016).

Fibronectin mediates many cellular interactions with the ECM, playing an important part in cell adhesion, migration, growth and differentiation. It is determinant for vertebrate development and is mostly synthesized by osteoblast precursors and mature bone cells; it can also be produced at distant sites (such as the liver) and enter the systemic circulation. Some studies suggested that only circulating fibronectin exerts effects on the bone matrix (Young, 2003; Bentmann et al., 2010). Fibronectin binds to collagen and may act as an extracellular scaffold, facilitating interactions of BMP1 with substrates (Huang et al., 2009). Fibronectin may also be vital for the osteogenic differentiation of mesenchymal cells (Linsley et al., 2013; Kang et al., 2017).

Osteonectin or SPARC (Secreted Protein Acidic and Rich in Cysteine) is secreted by osteoblasts during bone formation and it is one of the most abundant non-collagenous proteins in the bone matrix. Osteonectin is a regulator of bone mineralization; its attachment to collagen can inhibit or promote mineral formation. It interacts also with apatite through its N-terminal domain, inhibiting crystal growth (Matlahov et al., 2015). Osteonectin knockout mice suffer from osteopenia due to osteoblasts and osteoclasts defective function and low bone turnover. Changes in the osteonectin encoding gene have also been linked to idiopathic osteoporosis and osteogenesis imperfecta (Rosset & Bradshaw, 2016).

Thrombospondin-2 (TSP-2) is another matricellular protein that also exerts its effects on osteoblast proliferation and function, being involved in MSCs adhesion and migration; it has also influence on angiogenesis and tumour growth and metastization (Delany et al., 2000; Delany & Hankenson, 2009; Wang et al., 2019). TSP-2 likely participates in bone remodelling, since it promotes osteoclastogenesis through the RANKL-dependent pathway (Wang et al., 2019).

Bone sialoprotein (BSP) is a highly glycosylated and sulphated phosphoprotein that is found almost exclusively in mineralized connective tissues (Ganss et al., 1999). BSP knockout mice have higher trabecular bone mass and reduced amounts of cortical bone; they also present a very low turn-over. BSP defective mice maintain unloading bone response, as opposite to OPN knockout mice (Malaval et al., 2008). The absence of BSP also leads to changes in the growth plates, decreased bone length and delayed ossification (Holm et al., 2015). BSP and OPN are part of the Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLING) family and recent studies suggest the interplay in between these proteins is determinant in bone biology (Bouleftour et al., 2019).

Proteoglycan (PG) encoding genes are expressed in skeletal and non-skeletal tissues but with stronger expression in bone, joints and liver. PGs are a large family of molecules and perform many biological functions. PGs help to structure bone by mediating collagen secretion and fibril organization; they also act as mineralization inhibitors. PGs also modulate cytokines and growth factors biological activity in bone (Lamoureux et al., 2007). In bone, PrG4 gene expression is under control of PTH (Novince et al., 2012).

From the reviewed above, it becomes clear that non-collagenous and collagen matrix proteins are fundamental for bone morphology and material properties, interacting with each other and with cells, and responding to stimuli generated locally or systemically. It is also evident that matrix components have a multiplicity of functions. The role of a molecule is modulated by changes in its structure and by interactions with other substances.

2.2.2 Bone cell population

Mature bone contains three core cell populations: osteoblasts, osteocytes and osteoclasts.

2.2.2.1 Osteoblasts

Osteoblasts arise from MSCs, sharing a common background with chondrocytes, myoblasts and fibroblasts. Osteoblasts differentiate under the influence of a variety of hormones, cytokines and the local mechanical environment (Nakamura, 2007). These cells, when active, are cuboidal/round (Fig.4), with specific features consistent with their secretory functions, such as prominent Golgi complexes and endoplasmic reticulum (with multiple vesicles and vacuoles); these are even more evident during matrix secretion and early stages of mineralization (Palumbo, 1986).

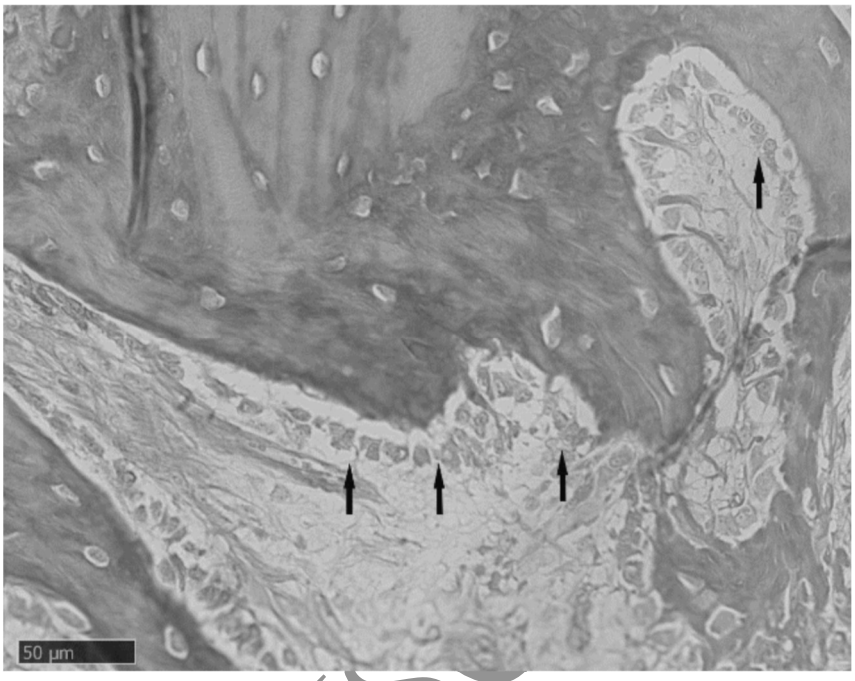

Fig. 4 Osteoblasts are cuboidal cells that when actively deposing matrix on bone surfaces (microphotograph, decalcified sheep bone section, Mason trichrome, magnification 40X; black arrows point line of active osteoblasts). When quiescent, osteoblasts appear as flat bone lining cells.

Osteoblasts can also remain on bone surfaces as flat bone lining cells, in a quiescent state, with few apparent cell organelles. During osteoblast maturation process there are increased levels of expression of pro-collagen, osteopontin and osteocalcin; bone sialoprotein seems to be more strongly expressed at intermediate phases of differentiation (Bellows et al., 1999; Bellows & Herschel, 2001). Osteoblast differentiation is impaired when gap junctions are inhibited, suggesting communication to neighbouring cells is essential for differentiation (Schiller et al., 2001). Osteoblasts produce non-mineralized matrix – osteoid - that becomes gradually mineralized, wherein they become trapped and some differentiate into osteocytes. Runx2 induces the expression of major bone matrix protein genes in vitro. Runx2 expression is up-regulated in preosteoblasts, being maximal in immature osteoblasts and down-regulated in mature osteoblasts. Although Runx2 is weakly expressed in undifferentiated mesenchymal cells, it induces their osteogenic commitment (Komori, 2019). Once Runx2 is activated, cells undergo the three stages of differentiation, with synthetization of different molecules: in stage 1 the cells proliferate and express fibronectin, collagen, TGFβ receptor 1, and osteopontin; during stage, osteoblast will differentiate and act on the extracellular matrix through alkaline phosphatase and collagen; at stage 3 the osteoblast will

assume its characteristic cuboidal shape and secrete significant amounts of osteocalcin. Osteocalcin will promote matrix mineralization (Rutkovskiy et al., 2016). Osteoblast differentiation is influenced by $1,25(OH)_2D_3$ and mechanical stimuli, amongst other factors (van der Meijden et al., 2016).

2.2.2.2 Osteocytes

Osteocytes are the most abundant cells of bone, comprising more than 90% of the osteoblast lineage and contributing to bone formation and resorption (van Bezooijen et al., 2004; Bonewald et al., 2007). They are fully differentiated osteoblasts embedded in the mineralized matrix, inside the osteocytic lacunae. Lacunae are located between the lamellae and connected with surrounding lacunae by a canalicular system (Fig. 5). Osteocytes have long dendritic cell processes (50 to 60 by cell) that lay within the canaliculi. The extremities of the cell processes connect osteocytes amongst themselves and allow contact with osteoblasts and bone lining cells (Carter & Beaupré, 2001; Knothe Tate et al., 2004; Jiang et al., 2007). The resulting functional syncytium shares a common environment (Knothe Tate, 2003).

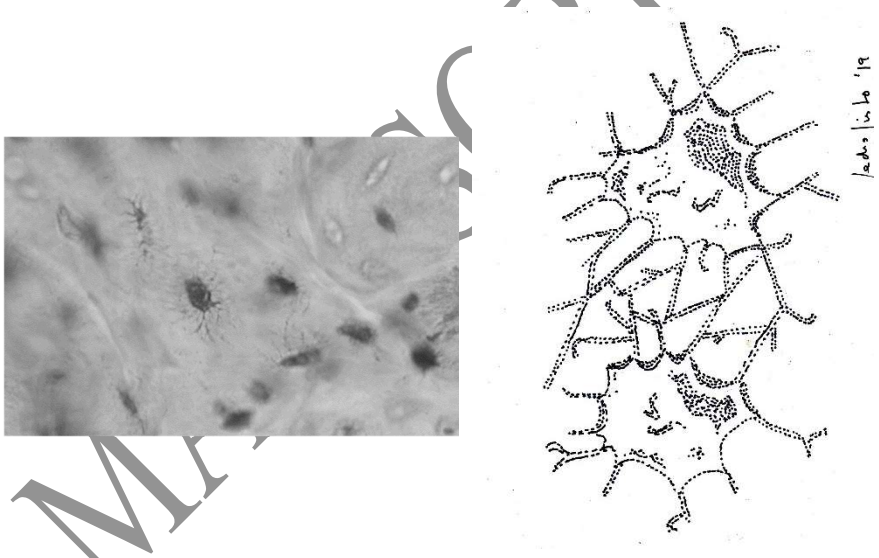

Fig. 5 Detail of microphotograph of an undecalcified bone section of sheep tibia, Giemsa-Eosin, on the left, showing osteocytes (Giemsa-Eosin, 40X magnification and 200% zoom; slide digitalized using Nanozoomer SQ, Hamamatsu Photonics, Portugal). The canaliculi where cell processes run are observable. The image on the right illustrates a simplified version of the resulting three-dimensional syncytium.

Osteocytes have no matrix secretion functions; however, they are responsible for sensing changes in the bone structure and commanding bone remodelling.

Pre-osteoblasts and osteoblasts are less responsive to fluid shear stress than osteocytes. Mechanosensitivity seems to increase during differentiation. However,

osteoblasts are able to modulate their response according to the mechanical stimuli intensity (Sommerfeldt & Rubin, 2001). Osteocyte functions include mechanosensing and maintaining bone matrix (Burger & Klein-Nulend, 1999; Mullender et al., 2004). The sensation of electrical signals may be one of the functions of osteocytes, and electrical signals mediated by osteocytes may regulate the cell behaviour in bone tissue (Huang et al., 2008). Flexoelectric fields are generated by fractures in the bone mineral and may be large enough to induce osteocyte apoptosis and initiate bone remodelling (Vasquez-Sancho et al., 2018).

The same mechanical stimulus may cause a different response in osteocytes according to their cell body shape (van Oers et al., 2015). Recently, a study reports that osteocyte plasma membrane disruptions, caused by mechanical loading, act as triggering mechanosensing events, both in vitro and in vivo (Yu et al., 2018).

Osteocytes early response to mechanical loading results in vesicular ATP release by exocytosis, tuned according to the magnitude of the stimulus (Kringelbach et al., 2015). Mechanical stimulation of osteocytes also causes fluctuations in intracellular calcium levels; these are responsible for calcium-dependent actin contraction and release of extracellular vesicles containing bone regulatory proteins (Morrell et al., 2018). In fact, osteocytes respond to mechanical stimuli by producing various messenger molecules, such as nitric oxide and prostaglandins, namely prostaglandin E2 (PGE2) (Klein-Nulend et al., 1998; Cherian et al., 2003; Mullender et al., 2004). This response is dependent on the function of stretch-activated calcium channels (Rawlinson et al., 1996), although reserves of intracellular calcium also contribute (Morrell et al., 2018). PGE2 has anabolic effects, stimulating osteoblast activity and new bone formation (Jee et al., 1990). Nitric oxide inhibits bone resorption, by suppressing osteoclast formation and increasing the expression of osteoprotegerin (Kasten et al., 1994; Fan et al., 2004).

The lifespan of osteocytes is highly variable and likely associated with the rate of bone remodelling, depending on mechanical and environmental factors such as hormones; osteocytes apoptosis may be inhibited or induced by a variety of physiological and pathological conditions. Osteocyte apoptosis may be induced by biological effectors such as hormones, without being accompanied by increased osteoclastogenesis (Tomkinson et al., 1997; Lee et al., 2004; Plotkin et al., 2005; Hirose et al., 2007; Jilka et al., 2013).

Young osteocytes are polarized toward the mineralization front, just like osteoblasts are, with the nucleus remaining close to vessels (Palumbo, 1986). As lamellar bone matures, the osteocytes tend to spread their processes perpendicularly to the longitudinal axis of trabeculae and long bones and appear as flattened cells. In immature bone, plump osteocytes with randomly distributed processes predominate (Hirose et al., 2007). Osteocyte density is closely related to bone architecture and thus to its mechanical behaviour (Metz et al., 2003).

Ageing has been correlated with smaller canaliculi, in lower numbers per lacuna, leading ultimately to reduced mechanosensitivity in the aged individual (Okada et al., 2002, Milovanovic et al., 2013).

2.2.2.3 Osteoclasts

Osteoclasts are multinucleated cells and belong to the same lineage as macrophages and monocytes (Fig. 6). Like macrophages, osteoclasts are able to merge and form multinucleated cells and to phagocytise (Rubin & Greenfield, 2005). The cell precursor may differentiate into either an osteoclast or a macrophage. The differentiation path depends on the progenitor cell being exposed to a receptor activator of several ligands (Receptor Activator of Nuclear factor κB Ligand - RANKL, osteoprotegerin and osteoclast differentiation factor - ODF) or to colony-stimulating factors related to immune system (Nakagawa et al., 1998; Asagiri & Takayanagi, 2007; Takayanagi, 2008).

The osteoclast presents distinctive functional features:

- osteoclasts can attach firmly to the bone surface, isolating the area under the cell membrane from its surroundings; the membrane domain responsible for the isolation of the resorption site is called sealing zone (Marchisio et al., 1984; Väänänen & Horton, 1995);
- osteoclasts acidify the mineral matrix by the action of protons pumps at the ruffled border membrane, a resorbing organelle; the lowering of the pH causes the dissolution of the hydroxyapatite crystals (Baron et al., 1985; Blair et al., 1989; Rousselle & Heymann, 2002);
- osteoclasts are capable of synthesizing and secreting enzymes such as tartrate-resistant acid phosphatase (TRAP) and cathepsins in a directional manner; the proteases secreted by osteoclasts cleave the organic matrix; through the combined action of lysosome enzymes, matrix metalloproteinases and the pH reduction, bone is resorbed (Littlewood-Evans et al., 1997; Vääräniemi et al., 2004);
- osteoclasts can phagocytise the resultant organic debris and minerals, removing them from the resorption lacunae, through a transcytosis process (Salo et al., 1997; Yamaki et al., 2005).

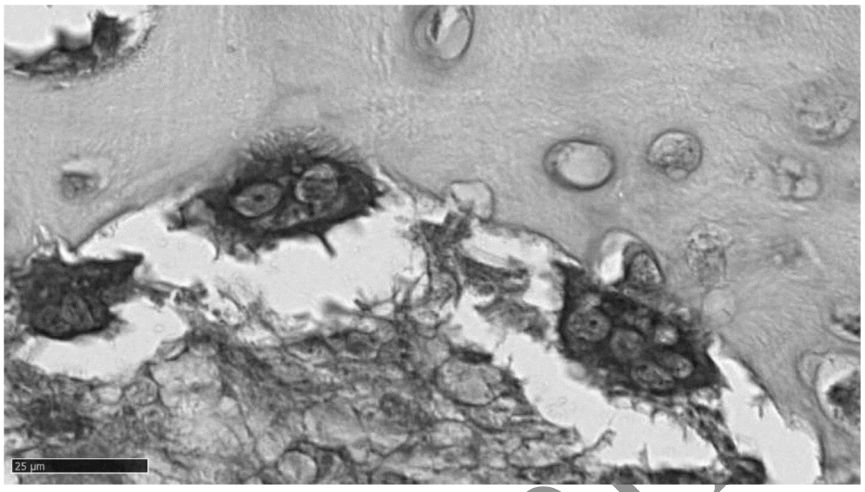

Fig. 6 A microphotograph of TRAP positive osteoclasts firmly attached to the bone surface. The ruffled border membrane is visible in direct contact with bone. This is the resorbing organelle; along its enlarged ruffled contact surface, proton pumps lower the local pH, dissolving hydroxyapatite.

The bone resorption process begins with differentiation and recruitment of osteoclast precursors, which merge and originate matured multinucleated bone-resorbing osteoclasts. Bone resorption begins when the osteoclast attaches to the mineralized bone matrix through the interaction of integrins with matrix proteins, like osteopontin and bone sialoprotein, previously laid down by osteoblasts (Väänänen & Horton, 1995).

2.3 Regulation of bone metabolism (modelling/ remodelling)

The bone cell populations are responsible for bone remodelling and repair. These processes are regulated systemically by hormones, neuropeptides and other mediators and locally by cytokines and growth factors (Harada and Rodan, 2003; Karsenty et al., 2009).

2.3.1 Parathormone (PTH), Vitamin D and calcitonin:

The bone mineral metabolism (calcium and phosphorus) is regulated by parathormone (PTH), calcitonin, FGF23 and vitamin D.

PTH is a peptide hormone produced by the parathyroid glands in response to low levels of extracellular ionized calcium, detected by specific cell-surface calcium-sensing receptors located in the parathyroid glandular tissue. High levels of PTH increase of the number of osteoclasts, and trigger resorption of bone matrix, with consequent release of calcium phosphate and increasing calcemia. This mechanism has developed as a protection against acute hypocalcemia. Inversely, low levels of PTH cause the elevation of osteoblast numbers. PTH also acts on osteoblasts' receptors, stimulating proliferation and differentiation and inhibiting apoptosis PTH

also acts on osteoblasts' receptors, stimulating proliferation and differentiation and inhibiting apoptosis (Siddiqui & Partridge, 2016). PTH also regulates kidney function by impairing phosphate reabsorption and promoting its excretion, by stimulating calcium reabsorption and up-regulating a hydroxylase enzyme (CYP27B1), thus promoting 1,25(OH)2 vitamin D3 synthesis (Murayama et al., 1998).

Circulating hormonal metabolite, 1α,25-dihydroxy vitamin D3 (1,25(OH)2D3) enhances several physiological functions, including intestinal calcium and phosphate absorption, bone phosphate and calcium resorption, and renal calcium and phosphate reabsorption, which results in a rise in the blood calcium and phosphate, required for bone passive mineralization of unmineralized bone matrix to occur (Haussler et al., 1998; Saini et al., 2013). Additionally, 1,25(OH)2D3 stimulates differentiation of osteoblasts and the expression of several bone proteins, like bone-specific alkaline phosphatase, osteocalcin, osteonectin, osteoprotegerin, and other cytokines; and influences the proliferation and apoptosis of other bone cells, including hypertrophic chondrocytes (Clarke, 2008). This may help explain why endogenous PTH levels can have anabolic and catabolic effects and are associated with differential skeletal effects on cortical and trabecular bones (Hong et al., 2019).

Calcitonin is produced by parafollicular cells of the thyroid, in direct response to extracellular calcium, through the same sensor that regulates the production of PTH. It inhibits matrix resorption, promotes calcium and phosphate excretion, thus reducing calcium and phosphate serum levels; calcitonin inhibits osteoclast mobility and the secretion of proteolytic enzymes (Boissy et al., 2002; Hadjidakis & Androulakis, 2006).

2.3.2 Growth hormone (GH):

Growth hormone or somatotropin is secreted in pulses by the anterior pituitary gland, inducing bone longitudinal growth (Isaksson et al., 1982). It also induces organs such as the liver and the skeleton to synthesize somatomedins that influence growth, such as insulin-like growth factor 1 (IGF-1) and 2 (IGF-2) (Ohlsson et al., 1998). The chondrocytes in the epiphyseal plate are stimulated not only by IGF1 and IGF2 but also directly by GH; proliferative and hypertrophic chondrocytes also secrete IGFs; IGF-1 acts inhibiting further GH secretion (Wu et al., 2015; Ranke & Wit, 2018).

According to Ohlsson et al. (1998), GH action in bone remodelling follows a “biphasic model”: initially it increases bone resorption, causing bone loss, followed by a phase of increased bone formation. When bone formation is more stimulated than bone resorption (transition point), the bone mass increases. A net increase of bone mass will be seen after 12–18 months of GH treatment in GH deficient adults (Kuzma et al., 2014). GH increases bone growth, by increasing both periosteal and endocortical bone formation, bone mineral content (BMC) and bone mineral density (BMD). GH acts synergistically with PTH to increase bone growth and bone

formation, bone density and mass and to decrease bone resorption (Guevarra et al., 2010).

2.3.3 Insulin and insulin-like growth factors (IGF-1 and IGF-2):

IGF-1 stimulates chondrocyte proliferation in the growth plate, thus playing a crucial role in longitudinal bone growth (Lupu et al., 2001). It is also involved in the formation of trabecular bone (Zhang et al., 2002). Insulin and IGF-1 have anabolic effects over the osteoblast and promote bone development, mainly through the activation of Akt and ERK signalling pathways; also, IGF-1 is capable of inducing osteoblasts in vivo proliferation whilst inhibiting the gene expression of osteocalcin, a marker for differentiating osteoblasts; insulin enhances osteocalcin expression but has no effect on osteoblast proliferation (Zhang et al., 2012). Additionally, insulin indirectly enhances Runx2 expression, a regulator of osteoblast differentiation (Fulzele et al., 2010; Zhang et al., 2012). A study with insulin-deficient type I diabetic mice showed that these mice presented a decreased expression of Runx2 and the Runx2-regulated genes, like osteocalcin and collagen type I, and a secondary decrease in bone formation. Bone loss was restored after insulin treatment, which increased Runx2 expression and the expression of related genes (Fowlkes et al., 2008).

Likewise, IGF-2 potentiates BMP-9-induced osteogenic differentiation and bone formation (Chen et al., 2010) through PI3K/AKT signalling. Moreover, a recent study in mice aortas showed that IGF-2 induces the expression of miR-30e, in a feedback loop. miR-30e is a major down-regulator of osteogenic differentiation of MSCs and smooth muscle cells (Ding et al., 2015).

2.3.4 Sex steroids (oestrogen and testosterone):

Bone metabolism is strongly influenced by sex steroids. Oestrogen is an important regulator of skeletal development and homeostasis, both in men and women, exerting direct and indirect effects on the skeleton (Turner et al., 1994; Prince et al., 1994; Khosla & Monroe, 2018). Indirectly it influences, for example, the calcium intestinal absorption (Liel et al., 1999; ten Bolscher et al., 1999) and secretion (Draper et al., 1997), and the calcium renal excretion; oestrogen also influences the secretion of PTH (Väänänen et al., 2005; Robinson et al., 2009). Oestrogen maintains bone homeostasis by inhibiting osteoblast and osteocyte apoptosis (Tomkinson et al., 1997; Kousteni et al., 2002; Emerton et al., 2010) and it inhibits osteoclast formation and activity, inducing osteoclast apoptosis (Hughes et al., 1996; Rodan & Martin, 2000; Faloni et al., 2007; Faloni et al., 2012; Khosla et al., 2012). Oestrogen deficiency causes bone loss and osteoporosis (Riggs et al., 1998). Androgens are also important to bone homeostasis. However, their role is likely more important during growth and contributes, via the GH/IGF system, to bone formation at the periosteum (Almeida et al., 2016). Androgens contribute to the maintenance of cancellous bone mass and integrity, regardless of age or gender (Compston et al., 2001; Vanderschueren et al., 2004). Androgen-deprivation therapy has negative effects on bone mineral density; these effects can be partially delayed by exercise, in the lumbar vertebrae but not in the hip (Taaffe et al., 2019).

2.3.5 Thyroid hormones:

The skeleton is a target-tissue for thyroid hormones, namely for thyroid hormone 3,5,3′-L-triiodothyronine (T3). Thyroid hormones influence bone growth during early development and adult bone turnover and maintenance. They act both directly, by stimulating bone resorption and formation and indirectly, by enhancing the effects of growth hormone over tissues. Hypothyroidism causes impaired bone formation and growth delay; thyrotoxicosis is a recognized cause of secondary osteoporosis and abnormal thyroid hormone signalling has been recognized as an osteoarthritis' risk factor (Bassett & Williams, 2016). T3 stimulates osteoblast proliferation and differentiation, with bone matrix secretion, modification, and mineralization.Thus, bone turnover is increased by thyroid hormones, which is confirmed by increased biochemical markers of bone turnover, such as osteocalcin and bone-specific alkaline phosphatase (Harvey et al., 1991; El Hadidy et al., 2011; Waring et al., 2013), and therefore bone loss can occur (Britto et al., 1994; Hadjidakis & Androulakis, 2006). Thyroid stimulating hormone (TSH), produced by the hypophysis, has direct effects on bone turn-over (Abe et al., 2003) and TSH receptors have been found on osteoblasts and osteoclasts, although available data does not allow conclusions on whethert TSH inhibits, increases, or does not affect osteoblast differentiation and function (Bassett & Williams, 2016). Still, recombinant TSH showed antiresorptive effects in ovariectomized rats (Abe et al., 2003; Sun et al., 2008) and lower TSH levels – with no apparent association with free T4 levels – have been related to hip fracture risk, supporting the idea that TSH effect on the skeleton may be independent on free T4, though its action on dedicated membrane receptors can be up-regulated by modulators (Waring et al., 2013; Neumann et al., 2018).

2.3.6 Leptin ("satiety" hormone):

Leptin is produced mainly in adipose tissue and it is a regulator of food intake and energy expenditure through its effects on the central nervous system (CNS). Its influence in bone metabolism probably follows two pathways: a central pathway, activating the sympathetic nervous system that inhibits bone formation, and a peripheral pathway promotes bone formation through leptin receptors on osteoblastic cells (Shi et al., 2008; Chen & Yang, 2015). Leptin inhibits osteoclast generation (Holloway et al., 2002), promotes the decrease in cancellous bone and increase in cortical bone, thus enhancing bone enlargement (Elefteriou et al., 2004; Hamrick & Ferrari, 2008); it also increases osteoblast number and activity, acting primarily through the peripheral pathways (Turner et al., 2013). Another study showed that leptin increases bone mineral content and density, especially at the lumbar spine (Mantzoros et al., 2011). However, in the ovine foetus, leptin infusion caused increased femur porosity and connectivity density, and vertebral trabecular thickness whilst leptin receptor antagonist infusion decreased trabecular spacing and increased trabecular number, degree of anisotrophy, and connectivity density in the lumbar vertebrae; effects differed in females and males (DeBlasio et al., 2018). Leptin also increases the expression of IGF-1 receptor and IGF-1 receptor messenger (Maor et al., 2002). During infancy and childhood, leptin and IGF-1 were

associated with body composition in preterm-born children. The same study also describes leptin association with bone parameters in early infancy, but not in childhood (Ruys et al., 2018). These results suggest leptin role on bone metabolism and architecture may vary with gender, age and interaction with other hormones and factors. Leptin is also a key up-regulator of FGF23 secretion (Tsuji et al., 2010) and it has been described as a direct enhancer of parathormone secretion (Lopez et al., 2016).

2.3.7 Bone Morphogenetic Proteins (BMPs):

BMPs are a group of 15 growth factors also known by cytokines, which belong to the transforming growth factor β (TGF-β) superfamily, with the ability to induce the formation of bone (Urist et al., 1965) and cartilage (Kobayashi et al., 2005). BMPs play a major role in the regulation of osteoblast lineage-specific differentiation and later bone formation (Beederman et al., 2013). Alterations in BMPs activity are often associated with a great variety of clinical pathologies, like skeletal and extra-skeletal anomalies, autoimmune, cancer, and cardiovascular diseases (Rahman et al., 2015). BMPs crosstalk with several other major signalling pathways, e.g. Wnt, Akt/mTOR, miRNA, among others, having Runx2 as a key integrator (Lin & Hankenson, 2011; Rahman et al., 2015). Among all BMPs, BMP9 has stronger osteogenic inductive activity over MSCs (Kang et al., 2004; Kang et al., 2008; Beederman et al., 2013); BMP9 also acts synergistically with TGF-β and GH to enhance bone formation (Li et al., 2012; Huang et al., 2012; Rahman et al., 2015). In addition to BMP9, other BMPs also have shown the ability to induce osteogenesis in vivo, such as BMP2, BMP6 and BMP7 (Franceschi et al., 2000; Jane et al., 2002; Cheng et al., 2003), with recombinant human-BMP2 and -BMP7 already being commercialized with the purpose of enhancing bone healing (Carreira et al., 2014). However, recent studies indicate the existence of age-related differences in BMP2-mediated bone regeneration, including relative dose sensitivity (Cheng et al., 2019). Contrariwise, BMP3 is known to be a negative regulator of bone formation and BMP4 has been shown to decrease trabecular bone formation in a murine model (Kang et al., 2004; Holien et al., 2018).

2.4 Bone remodelling and cell interchange

Healthy bone, both cortical and trabecular, is continuously remodelling, a dynamic process with bone resorption and formation. Bone remodelling is modulated by mechanical loading, blood calcium levels and a wide range of paracrine and endocrine factors.

The bone remodelling process depends on the coordinate actions of osteoblasts, osteoclasts, osteocytes and osteoblast-derived bone lining cells, along with other cells, such as macrophages and immune cells. The ensemble constitutes the “Basic Multicellular Unit” (BMU) or “Bone Remodelling Unit” (BRU). In the BMU, the amount of bone lysis achieved by osteoclasts is equal to the amount of bone produced by osteoblasts. The balance between osteoblastic and osteoclastic activity

is known as coupling. Frost proposed that bone longitudinal growth, modelling, and BMU-based remodelling activities were modulated by a "mechanostat", a mechanism modulating bone mass in function of mechanical use, in which BMUs would play a central role, along with bone longitudinal growth and modelling (bone formation). Bone modelling was thus considered as an adaptative response to overloading and remodelling as a response to underloading, with given strain setpoints for each process (Frost 1987).

Osteoclasts and osteoblasts within the BMU may function under the control of other cell types, since osteoblasts and osteoclasts may perform their functions in the absence of each other (Corral et al., 1998; Kong et al., 1999). Cells from the osteoblast lineage express receptors for cytokines and other local secreted factors that stimulate osteoclast formation (Suda et al., 1999). The BMU can be inhibited by old age, drugs, endocrine, metabolic or inflammatory diseases.

Regardless of the triggering stimulus, osteoclast formation depends on RANKL. Osteoblasts express membrane-bond RANKL and this regulatory molecule interacts with a receptor (receptor activator of nuclear factor-κB - RANK), expressed on the surface of osteoclast precursors. The RANK activation by RANKL is essential for fusion of the osteoclast precursor cells and osteoclast formation (Miyamoto & Suda, 2003).

Both down-regulation and up-regulation of RANKL expression by osteoblasts under similar mechanical stimulation have been described (Fan et al., 2006; Kreja et al., 2008). Osteoblasts subjected to different mechanical stimuli respond by an increase in RANKL-bound and a decrease in soluble RANKL secretion (Kim et al., 2006).

The RANKL/ RANK coordinated effects can only be understood by adding osteoprotegerin to the axis. Cells from osteoblastic lineage produce osteoprotegerin (OPG). OPG is soluble and blocks the interaction between RANKL and RANK, acting as a decoy receptor for RANKL. OPG thus inhibits osteoclast formation and induces osteoclast apoptosis (Liu et al., 2015). Osteoblasts, in addition, secrete macrophage colony stimulating factor-1 (M-CSF-1); M-CSF-1 promotes osteoclast precursor proliferation and RANK expression (Arai et al., 1999; Romas et al., 2002). Osteoblast-like cells cultures mechanically stimulated may respond by a decrease in the production of OPG, without a change in the RANKL production, with a consequent increase in the ratio of RANKL/OPG. This could translate into increased bone remodelling. However, subjecting osteoclast-like cells to the same mechanical stimuli regimen, decreased TRAP and a period of stimulation of one minute at 0.3 Hz frequency, a decrease in cell fusion and resorption activity was observed (Kadow-Romacker et al., 2009).

RANKL expression by osteoblast-lineage cells is enhanced when microdamage within the bone matrix occurs. Microdamage may occur under physiological bone loading and in pathological conditions. The presence of microcracks is sensed by osteocytes and may induce osteocyte apoptosis; osteocyte apoptosis may also be induced by disuse and is closely correlated with higher bone remodelling levels

(Mori & Burr, 1993; Bentolila et al., 1998; Verborgt et al., 2002; Noble et al., 2003; Mann et al., 2006; Martin, 2007, Jilka et al., 2013).

Pulsating fluid flow (PFF)-treated osteocyte cultures conditioned the culture medium, inhibiting osteoclast formation and decreasing in vitro bone resorption. These effects have not been detected in the medium from PFF-treated fibroblast cultures (Tan et al., 2007). In osteocytes subjected to PFF, nitric oxide is involved in the up- and down-regulation of at least two apoptosis-related genes (Bcl-2 and caspase-3, with antiapoptotic protective and pro-apoptotic functions, respectively) (Tan et al., 2008). Nitric oxide (NO) is a second messenger molecule produced in response to mechanical stimulation of osteoblasts and osteocytes, and other cell types such as endothelial cells, with a large variety of biological functions (Smalt et al., 1997; Zaman et al., 1999; Rössig et al., 2000; van'T Hof, 2001).

Osteocytes, thus, regulate osteoclastogenesis and osteoclast activity through soluble factors and messenger molecules.

Other pathways are relevant for osteoblasts, osteocytes and osteoclasts interweaved regulation, such as the Notch signalling pathway. In osteocytes, the Notch receptors activation induces OPG and Wnt signalling, decreasing cancellous bone remodelling and inducing cortical bone formation (Canalis et al., 2013). Wnt/Lrp5 signalling in osteocytes has been considered as a key pathway for bone response to loading (Bullock et al., 2019).

2.5 Bone mechanotransduction

Bone mechanotransduction, essential in health and disease states, is not yet fully understood. The elements involved in transduction include the ECM, cell-cell adhesions, cell-ECM adhesions, cell membrane components, specialized surface processes, nuclear structures and cytoskeleton.

2.5.1 The cell membrane elements, cell-cell and ECM-cell adhesions

Cell membrane-associated mechanotransduction mechanisms depend on the integrity of the phospholipid bilayer. Mechanotransduction pathways are disrupted if membrane cholesterol is depleted, inhibiting the response to hydrostatic and fluid shear stress (Ferraro et al., 2004; Xing et al, 2011). Cytoskeleton actin polymerization and assembly is influenced by membrane cholesterol levels (Klausen et al., 2006; Qi et al., 2009). However, it has been proposed that actin polymerization during synaptic vesicle recycling is influenced by vesicular cholesterol, but not plasma membrane cholesterol, as suggested by a study wherein the inhibition of actin polymerization by the extraction of vesicular cholesterol resulted in the dispersal of synaptic vesicle proteins (Dason et al., 2014). But even with a functional cell membrane, if integrin binding is impaired, actin cytoskeleton will not re-organize in response to shear stress (Radel & Rizzo, 2005). However, nanometer- to micron-sized tears, reparable defects in the cell plasma membrane

promote particle flux across the cell membrane, namely Ca^{2+} influx (Yu et al., 2018).

Integrins are cell adhesion receptors, heterodimers of non-covalently associated 18α and 8β subunits, in mammals, that can combine to generate 24 different receptors with different binding properties and different tissue distribution (Hynes et al., 2002; Barczyk et al. 2010). These subunits possess an extracellular portion with several domains, able to bind to large multi-adhesive ECM molecules, which in turn bind to other ECM molecules, growth factors, cytokines and matrix-degrading proteases (Barczyk et al., 2010). Integrins were first acknowledged as bridging the ECM and the cell cytoskeleton, including the actin cytoskeleton but also the intermediate filament network, essentially vimentin and laminin (Nievers et al., 1999). Cells use multiple mechanisms to sense and respond to mechanical stress applied to integrins (Matthews et al., 2006). Recruitment of vimentin has been shown to depend on integrin β3 subunits, underpinning the relationship between the various cytoskeletal elements and integrins (Bhattacharya et al., 2009). The cytoplasmatic portions of integrin β subunit bind to talin, which can also directly bind to vinculin and actin filaments (Cram & Schwarzbauer, 2004). On the other hand, integrin α4 subunit binds to paxillin (Brown et al., 1996), a protein that integrates sites of cell adhesion to the ECM.

Integrins allow communication between structures in the interior and outside of the cell, in a bidirectional way. The inside-out signalling turns the integrin extracellular domains into the active conformation. In the outside-in pathway, when an integrin binds to the extracellular ligand, it clusters with other bound integrins, forming focal adhesions, highly organized intracellular complexes; these are connected to the cytoskeleton. The focal adhesions integrate a range of different molecules, including the cytoplasmic portions of the clustered integrins, proteins of the cytoskeleton, and signalling molecules (Cram & Schwarzbauer, 2004; Geiger et al., 2009). Initial adhesions to substrates are characterized by punctuate areas at the limits of lamellipodia, usually known as focal complexes. Focal adhesions are the mature form of cell-matrix adhesion, with an elongated shape and are associated with bundles of actin and myosin (stress fibres). There is a specialized form of focal contact, in which integrin binds to fibronectin fibrils and tensin but with low levels of tyrosine kinases (Katz et al., 2000; El-Hoss et al., 2014). Most focal adhesions also contain several types of signalling molecules like tyrosine phosphatases and tyrosine kinases and adaptor proteins (Parsons, 1996; Yamada & Geiger, 1997; Geiger et al., 2009; Teo et al., 2013).

Matrix proteins may also modulate cell adhesion; connective tissue growth factor (CTGF), which is a matrix protein, enhances osteoblast adhesion (via $\alpha V\beta_1$ integrin) and cell proliferation, by inducing cytoskeletal reorganization and Rac1 activation (Hendesi et al., 2015). Another matrix protein – osteoactivin – also modulates osteoblast adhesion, differentiation and function, stimulating alkaline phosphatase (ALP) activity, osteocalcin production, nodule formation, and matrix mineralization (Moussa et al., 2014). α5β1 integrin interacts with its high-affinity ligand

CRRETAWAC, enhancing the Wnt/β-catenin signalling mechanism to promote osteoblast differentiation independently of cell adhesion (Saidak et al., 2015).

Cell adhesion and mechanical stimulation depend on integrin mediation (Carvalho et al., 1998). Forces applied to integrin receptors cause local adhesion proteins to be recruited and the cell adapts by making the integrin-cytoskeleton linkages more rigid; myosin II contraction makes the cell apply tension to the substrate (Riveline et al., 2001). Different signalling pathways are triggered by sensed stress through integrin receptors. Sequential expression of integrin ligands (osteopontin, fibronectin and bone sialoprotein) in response to mechanical stimulation of osteoblasts has been described (Carvalho et al., 2002). Bonds between integrin and ligands become stronger in the presence of cell tension (Friedland et al., 2009).

Osteocytes are highly specialized in their interaction with ECM; osteocyte cell bodies express β1 integrins while cell processes express β3 integrins, the latter in a punctuate distribution (Phillips et al., 2008; McNamara et al., 2009; Litzenberger et al., 2009 Litzenberger et al., 2010). Thi et al. identified the cell processes as the mechanosensory organs in osteocytes (Thi et al., 2013). It has been demonstrated that integrin αVβ3 is essential for the maintenance of osteocyte cell processes and also for mechanosensation and mechanotransduction by osteocytes, by ATP release that triggers calcium signalling (Haugh et al., 2015; Cabahug-Zuckerman et al., 2018). β1 integrins have been shown to regulate specific aspects of mechanotransduction, namely the cortical osteocyte response to disuse (Phillips et al., 2008). In osteoblasts, a mechanical load applied to β1 integrin subunit results in calcium influx (Pommerenke et al., 2002), independently from gap junctions (Saunders et al., 2001). Another study showed that ERK1/2 activation by strain prevented osteocyte apoptosis but required the integrin/cytoskeleton/Src/ERK signalling pathway activation (Plotkin et al., 2005).

Apart from integrin, other membrane proteins are responsible for conduction of mechanical stimuli. Cadherins, which connect to the cytoskeleton, also mediate force-induced calcium influx (Gillespie & Walker, 2001; Kazmierczak et al., 2007), and participate in the Wnt/β-catenin pathway (Marie & Hay, 2013). In osteoblasts, it has been suggested that GPI-anchored proteins may play an important role in mechanosensing, by demonstrating that the overexpression of GPI-PLD, an enzyme that can specifically cleave GPI-anchored proteins from cell membranes, inhibits flow-induced intracellular calcium mobilization and ERK1/2 activation in MC3T3-E1cells (Xing et al., 2011). Ephrins (ligands) and Ephs (receptors) contribute to cell-cell interactions between osteoclasts and osteoblasts, helping to regulate bone resorption and formation, and appear to be necessary for hMSC differentiation (Tamma & Zallone, 2012; Matsuo & Otaki, 2012). Lastly, another family of proteins – galectins – is also involved in regulating osteogenesis; for example, Gal-3, which is expressed both by osteocytes and osteoblasts, plays a significant role as a modulator of major signalling pathways, such as Wnt signalling, MAPK and PI3K/AKT pathways (Nakajima et al., 2016); Gal-8 induces RANKL expression by osteoblasts and osteocytes, osteoclastogenesis and bone mass reduction in mice (Vinik et al., 2015); and Gal-9 induces osteoblast differentiation through the

CD44/Smad signalling pathway in the absence of bone morphogenetic proteins (BMPs) (Tanikawa et al., 2010).

Gap junctions are transmembrane channels that connect the cytoplasm of adjacent cells. Only small metabolites, ions and signalling molecules like calcium and cAMP pass through these channels since the molecular weight must be lower than 1 kDa (Flagg-Newton et al., 1979; Steinberg et al., 1994). Gap junctions are essential for bone mechanosensation since in osteoblastic cells the PGE2 production induced by fluid flow is dependent on intact gap junctions; if these are disturbed, PGE2 production does not occur (Saunders et al., 2001; Saunders et al., 2003). Mice lacking Cx43 gap junctions in osteoblasts and/or osteocytes exhibit increased osteocyte apoptosis, endocortical resorption, and periosteal bone formation (Bivi et al., 2012).

2.5.2 Primary cilia

In different cell types, different structures ensure recognition of mechanical stimuli; kidney epithelial cells possess a single microvillar projection on their apical surface (primary cilia). A similar structure was described in osteoblasts and osteoblast-like cells (Myers et al., 2007; Delaine-Smith et al., 2014). Primary cilia originate in the centrosome and project from the surface of bone cells; its deflection during flow indicates that they have the potential to sense fluid flow. These cilia deflect upon application of 0.03 Pa steady fluid flow and recoil after cessation of flow (Xiao et al., 2006; Malone et al., 2007). In bone, primary cilia translate fluid flow into cellular responses, independently of Ca^{2+} flux and stretch-activated ion channels (Malone et al., 2007). It has been demonstrated in vitro that, apart from mediating the up-regulation of specific osteogenic genes, primary cilia are also chief mediators of oscillatory fluid flow-induced extracellular calcium deposition, thereby playing an essential role in load-induced mineral matrix deposition (Delaine-Smith et al., 2014). A study using knockout mice of Kif3a, which results in defective primary cilia, showed that primary cilia are essential for the ability of pre-osteoblasts to sense strain-related mechanical stimuli at a healing bone-implant interface, inducing osteoblast further differentiation (Leucht et al., 2013); using the same animal model, another study shown primary cilia were paramount for MSCs to sense mechanical signals and enhance osteogenic lineage commitment in vivo (Chen et al., 2016). Primary cilia must also be present in osteocytes for pulsed electromagnetic fields to inhibit osteocyte-mediated osteoclastogenesis and inhibit osteocyte apoptosis, modulate cytoskeletal distribution, and decrease RANKL/OPG expression (Wang et al., 2019).

Concerning osteocytes, there is still conflicting information regarding in vivo expression of cilia. Their role as mechanosensors depends on the type and number of cells with cilia, and on the local mechanical environment. The incidence of primary cilia in osteocytes has been described as of 4%; this may indicate that cilia function as mechanosensors on a selected number of cells or that cilia function in concert with other mechanosensing mechanisms (Coughlin et al., 2015).

2.5.3 The cytoskeleton

The cell cytoskeleton network is coupled to the ECM through specific transmembrane receptors. Integrins connect to the cytoskeleton through focal adhesions that gather actin-associated proteins such as talin, vinculin, paxillin and zyxin. Both paxillin and zyxin belong to a group of LIM domain structural proteins, which have been suggested as mechanoresponders responsible for regulating stress fibres assembly, repair, and remodelling in response to changing forces (Smith et al., 2014). Focused stresses applied to the surface of the cellular membrane are transferred across the network of cell adhesions, microfilaments and microtubules and affect distant cellular sites such as the mitochondria and nucleus, or the cell membrane on the opposite side. The transmission of strain towards the ECM stimulates structural changes at a higher organization level, making it stronger (Wang et al., 1993; Wang & Ingber, 1994).

The cell deformation in consequence of applied stress does not correspond to the predicted behaviour of an isotropic viscoelastic material; the interior of the cell, the cytoskeleton, are anisotropic. The intricate network of microtubules and microfilaments, how it spreads and is connected to the point of applied force, may result in structures away from the load application point to be further displaced than closer ones; displacements towards the origin of the compressive stimulus are also possible. Behaving in an anisotropic way, cells can respond to an external force according to its magnitude and direction (Hu et al., 2003; del Álamo et al., 2008; Silberberg et al., 2008). An intact cytoskeleton is necessary for the rendering of applied forces into mitochondria movements. Since mitochondria are semi-autonomous organelles, highly dynamic, the distress caused by mechanical stimuli exerts biological effects on their function (Silberberg et al., 2008), both in health and disease (Koike et al., 2015).

It is, therefore, logical that mechanical properties of the ECM affect the behaviour of cells from osteoblastic lineage, with mature focal adhesions and a more organized actin cytoskeleton associated with more rigid substrates, suggesting that controlling substrate compliance enables control over differentiation (Khatiwala et al., 2006) and that this influence on differentiation is independent of protein tethering and substrate porosity (Wen et al., 2014).

Other factors are determinant for cell fate. A recent in vitro study showed similar patterns in cell growth, differentiation, and gene expression in human osteoblasts and endothelial cells when implanted in two different ceramic scaffolds – β-tricalciumphosphate and calcium-deficient hydroxyapatite. These scaffolds had different chemical and physical characteristics, with results suggesting that the interaction between different cell types and scaffold materials is crucial for growth, differentiation, and long-term outcomes of tissue-engineered constructs (Ritz et al., 2016). It has also been highlighted the importance of surface roughness of the biomaterials in osteogenic differentiation, and the contribution of specific integrin subunits in mediating cell response to different materials (Olivares-Navarrete et al., 2015); additionally, the application of synthetic integrin-binding peptidomimetic ligands (αVβ3- or α5β1-selective) to a titanium graft enhanced cell adhesion,

proliferation, differentiation and ALP expression in vitro osteoblast-like cells, resulting in a higher mineralization on the surfaces coated with the ligands (Fraioli et al., 2015).

The biochemical nature of the substrate, its rigidity and spatial organization are recognized by cells through signalling from molecular complexes that are integrin-based.

In most anchorage-dependent cells, cell spreading on ECM is required for cell progression and growth; increasing cytoskeletal tension results in cell flattening, a rise in actin bundling and bucking of microtubules. Spread cells can transfer most of the load to the ECM.

The cell shape is influenced by how the cytoskeleton organizes its elements and it is determinant for cell function. For example, osteocyte morphology and alignment differ in two types of bone, fibula and calvaria, probably due to different mechanical loading patterns, which influence the cytoskeletal structure and thus cell shape (Vatsa et al., 2008). Also, osteocyte and lacunae morphology may vary in pathological bone conditions, and these morphological variations may be an adaptation to the differences in matrix properties and thus, different bone strain levels under similar stimuli (van Hove et al., 2009). Osteocyte morphology is characterized by long dendritic-like processes, cell shape also assumed by osteoblast MC3T3 cells cultured in 3D; however, differences in cytoskeleton elements in the processes of these two cell types may indicate differences in function; microtubules are predominant on osteoblasts' processes while actin ensures integrity of osteocytes' cytoplasmatic projections (Murshid et al., 2007). Osteocyte sensitivity to mechanical load applied to the microparticles varies between those attached to the cell bodies and the ones attached to the cell processes: a much smaller displacement of the second ones is needed to cause an intracellular calcium influx that rapidly propagates to the cell body; if local stimulus is applied to the cell body, the reaction is slower and a higher displacement is needed to cause the calcium transient (Adachi et al., 2009).

Osteoblasts, osteoid-osteocytes and mature osteocytes have different mechanical properties. The elastic modulus is higher in the cell periphery than in the perinuclear region; the elastic modulus in both regions decreases as bone cells mature. These differences in elastic modulus probably depend on the number of actin filaments, as it has been shown in other cell types. Furthermore, focal adhesion area is smaller in mature osteocytes, when comparing to osteoblasts. If peptides containing RGD sequence are added to culture medium, both the focal adhesion area and the elastic modulus of osteoblasts decreases whilst remaining unaffected in osteocytes (Sugawara et al., 2008).

2.6 Mechanotransduction mechanisms

The multitude of cellular structures, messenger substances, environmental factors and levels of organization of the organs involved in the mechanotransduction

mechanisms in distinct cells and tissues, makes it extremely complex to understand, predict and replicate how responses are composed at cellular, organ and living organism levels.

2.6.1 Strain, frequency and loading duration

Bone remodelling is influenced by strain magnitude, frequency and loading duration. Wolff developed mathematical equations for trabeculae orientation and thickness prediction according to load (Prendergast & Huiskes, 1995). Later, Turner enunciated three essential rules critical for bone remodelling (Turner, 1998):

1. Remodelling is determined by dynamic loading, not by static loading;
2. Short periods of loading quickly trigger a response; prolonging loading times any further diminishes the magnitude of bone cell response;
3. Bone cells have memory and accommodate to routine loading, diminishing the amplitude of the response triggered by a same repeated stimulus.

Increasing loading frequency increased strain-related bone deposition in vivo, whilst decreasing the threshold for osteogenesis and bone formation (Hsieh & Turner, 2001). Human osteoblasts subjected to strains varying from 0.8 to 3.2% respond to higher strain with increased expression of osteocalcin, type I collagen and Cbfa1/Runx2, and to lower strain magnitudes with an increase of alkaline phosphatase activity (Zhu et al., 2008).

Bone formation depends on strain magnitude (Mosley et al., 1997), along with the number of loading cycles at low frequencies (Cullen et al., 2001). Frost theorized that a minimum effective strain level was necessary to trigger bone formation, above 3000 micro-strain (Frost, 1987). Strain distribution is also paramount for skeletal adaptation. Unusual strain distribution will rapidly trigger an osteogenic response, as suggested by the extensive periosteal and endosteal bone proliferation described by Rubin & Lanyon (1984) in a study conducted in poultry. Rest periods between loading cycles also intensify osteogenic response (Srinivasan et al., 2007) and maximize cell response (Pereira & Shefelbine, 2014). During active exercise, peak strains in long bones may be high, but strains as low as 0.15% are enough to ensure osteoblast recruitment in vivo (Rubin & Lanyon, 1984). Human bone marrow stem cells show variable early osteogenic differentiation and gene expression accordingly with load and frequency regimen of cyclic hydrostatic pressure; osteogenic differentiation on the long term occurred under mechanical stimulation, independently of load magnitude and frequency, within the tested physiological ranges (Stavenschi et al., 2018).

The adaptation of cortical bone is correlated with frequency, although not linearly; the changes in geometry are more significant with higher frequency, with a plateau for frequencies past 10 Hz (Warden & Turner, 2004).

Other mechanisms apart from direct deformation of cells are involved in bone mechanical stimulation. Bone's canalicular system is filled with fluid. Simulation

of osteogenic load levels has produced higher shear stresses due to fluid displacement in the canaliculi. The fluid flows within the canalicular system wherein the osteocytes extend their cell processes, reinforcing osteocyte processes as the main mechanosensing organ in mature bone cells (Verbruggen et al., 2014). Multiple canaliculi intersect at points (canalicular joints); these occur with a density similar to that of lacunae and represent areas of enlarged space, with consequences on fluid flow variables such as fluid mass and velocity (Wittig et al., 2019). Microstructural changes associated with osteoporosis reduce interstitial fluid flow around osteocytes in the lacunar-canalicular system of cortical bone, impairing mechanosensation (Gatti et al., 2018).

As reviewed previously, shear stresses resulting from fluid flow cause calcium influx through mechanosensitive channels (Nauli et al., 2003; Praetorius et al., 2003). Calcium influx occurs is osteoblasts in response to oscillatory fluid flow (Saunders et al., 2001).

Fluid also carries electrically charged particles. The resulting fluid flow phenomena is common to other biological tissues but not limited to living structures. The fact that fluid flow changes interfacial chemistry has been recognised; the flow of fresh water along the surfaces disturbs the equilibrium of dissolved ions, changing the surface charge and the molecular orientation of the water at the interface (Waychunas, 2014). Likewise, when bone is deformed, a thin sheet of fluid with particles with charge opposite to that of the matrix and bone cells is formed (Gross & Williams, 1982); when a non-uniform mechanical load is applied to the bone structure, the ions in the fluid move away from the matrix. Therefore, the displacement of the electrically charged fluid creates an electrical field aligned with the fluid flow. This causes an electrical potential and the phenomenon is known as strain generated bone streaming potential and has been described in bone (Gross & Williams, 1982; Pienkowski & Pollack,1983; Frijns et al., 2005; Hong et al., 2008). The density of matrix fixed charges influences the magnitude of the generated streaming potential (Iatridis et al., 2003), so the mechanosensory ability along bone may vary and ultimately, influence dynamic stiffness.

2.6.2 Bone piezoelectricity and flexoeletricity

Fukada and Yasuda first described bone piezoelectrical properties, in the 50s. In dry bone samples submitted to a compressive load, an electrical potential was generated, an occurrence explained by the direct piezoelectric effect (Fukada & Yasuda, 1957). In connective tissues, such as bone, skin, tendon and dentine, the dipole moments are related to the collagen fibres, composed by strongly polar protein molecules aligned (Fukada & Yasuda, 1964; Elmessiery, 1981; Halperin et al., 2004).

Recently, it has been suggested that hydroxyapatite flexoelectricity is the main source of bending-induced polarization in cortical bone (Vasquez-Sancho et al., 2018).

The architecture of bone itself, with its aligned lamellae, contributes to the existence of potentials through the bone structure (Elmessiery, 1981).

Bone piezoelectric constants, i.e. the polarization generated per unit of mechanical stress, change with moisture content, maturation state (immature bone has lower piezoelectric constants when comparing to mature bone) and architectural organization (altered areas, such as bone neoplasia osteosarcoma, show lower values) (Marino & Becker, 1974). In dentin, piezoelectric constants are higher when moisture contents increase, also behaving anisotropically; tubule orientation determined piezoelectricity, stronger parallel to the tubules (Wang et al., 2007). Wet bone also behaves as a piezoelectric material (Fukada & Yasuda, 1957; Marino & Becker, 1974; Reinish & Nowick, 1975).

Bone piezoelectrical properties have risen interest, in the context of bone physiology and electromechanics. It has been related to bone remodelling mechanisms, and to streaming potential mechanisms (Ramtani, 2008; Ahn & Grodzinsky, 2009). Using a piezoelectric substrate and the piezoelectric converse effect were tested in vitro and in vivo with promising results, mechanically stimulating osteoblastic cells and bone, suggesting the potential for clinical application (Frias et al., 2010; Reis et al., 2012). The development of new synthetic scaffolds is a new emergent field for the bone tissue engineering industry. Hydroxyapatite/ barium titanate (Zhang et al., 2014) or polycaprolactone/barium titanate composites (Liu et al., 2019) with piezoelectric coefficients dependent on distribution and density of barium titanate particles aim to improve cell adhesion and differentiation. A wide range of biomaterials with piezoelectric properties, with potential application for bone regeneration, is available (Jacob et al., 2018). Damaraju et al. (2017) findings suggest mesenchymal cell differentiation in 3D piezoelectric scaffolds can be modulated by the voltage output (or streaming potential); lower voltage output scaffolds promoted chondrogenic differentiation and piezoelectric scaffolds with a high voltage output promoted osteogenic differentiation. Electromechanical stimulation also promoted improved differentiation when compared to mechanical load alone (Damaraju et al., 2017).

Due to the potential impact on therapeutic approaches to bone remodelling and healing, more and more research is being conducted on bioinspired approaches that consider piezoelectric bone properties.

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