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Dominik König Guerreiro

Bachelor of Science in Biomedical Engineering

Upregulation of Wnt regulators is associated with low bone mass in elderly Portuguese men and women

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Adviser: Dr. Rui Neves-Silva, Associate Professor,
Faculdade de Ciências e Tecnologias, Universidade NOVA de Lisboa

Co-adviser: Ana Maria Rodrigues, MD PhD, Assistant Professor
Nova Medical School

Examination Committee:

Chairperson: Prof. Dr. Jorge Carvalho Silva
Rapporteur: Prof. Dr. Saba Abdulghani Silva
Member: Prof. Dr. Rui Neves-Silva

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"Põe quanto És no Mínimo que Fazes"

Fernando Pessoa

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To conclude, I would like to thank the Portuguese Society of Rheumatology for funding this project.

Abstract

Bone biomarkers are chemical substances produced during the bone remodelling process that can provide beneficial information concerning bone metabolism. Recently, some studies highlighted the importance of Wnt signalling, a crucial pathway for osteoblast differentiation and a master bone mass regulator. In fact, serum levels of Dkk1 and SOST, which are negative regulators of Wnt signalling, increase with age and are associated with bone mass loss. Previous studies from the CEDOC group showed that, in fragility fracture patients, osteoblast terminal differentiation is impaired, which is associated with bone mechanical fragility. Therefore, it was hypothesized that serum Wnt regulators are associated to bone fragility and can constitute new markers for osteoporosis treatment decision.

In this dissertation, the association between bone gene expression of markers of osteoblast and osteoclast differentiation and of Wnt pathway regulators (Dkk1, Dkk2, SOST, WIF1 and sFRP1) with bone mineral density was analysed. Furthermore, the association between serum levels of bone biomarkers and Wnt regulators and bone mineral density was analysed as well. A set of 128 patients submitted to hip arthroplasty, aged above 40 years old, were evaluated from a clinical database. Linear regression analysis was performed to assess the above-mentioned associations. Associations within estimators were conducted to compute missing values. Stepwise regression was used with the Backward elimination process and bootstrapping was used to externally validate the models. Besides the bone biomarkers, four variables were included to the models, namely sex, rheumatoid arthritis, corticoid use and secondary osteoporosis.

Positive correlations were found between serum levels of Wnt regulators (P1NP, SOST and Dkk1) and BMD. With respect to the genetic expression of bone biomarkers, Dkk2 and sFRP1 were negatively associated with BMD, whereas Lrp6 and WIF1 were positively correlated. These results demonstrate that an upregulation of bone gene expression of Wnt regulators, namely some of the Wnt inhibitors, is associated with low bone mass. The low number of patients is a limitation and further studies need to be conducted in larger populations and with the inclusion of more bone biomarkers. This dissertation was conducted under the project ARIBOS, funded by the Portuguese Society of Rheumatology.

Keywords: Bone mineral density estimation; bone remodelling; Wnt pathway; Bone Biomarkers; Osteoporosis

Resumo

Os biomarcadores ósseos são substâncias químicas, produzidas durante o processo de remodelação óssea, que podem fornecer informações benéficas sobre o metabolismo ósseo. Recentemente, alguns estudos realçaram a importância da via Wnt, uma via crucial para a diferenciação dos osteoblastos e um regulador-chave de massa óssea. De fato, os níveis séricos de Dkk1 e SOST, que são reguladores negativos da via Wnt, aumentam com a idade e estão associados à perda de massa óssea. Estudos anteriores do grupo do CEDOC mostraram que, em pacientes com fraturas por fragilidade, a diferenciação terminal dos osteoblastos é prejudicada, o que está associado à fragilidade mecânica óssea. Assim, foi levantada a hipótese de que os reguladores séricos da via Wnt estão associados à fragilidade óssea e podem constituir novos marcadores para a decisão no tratamento da osteoporose.

Nesta dissertação, analisou-se a associação entre a expressão génica óssea de marcadores da diferenciação de osteoblastos e osteoclastos e de reguladores da via Wnt (Dkk1, Dkk2, SOST, WIF1 e sFRP1) com a densidade mineral óssea. Além disso, a associação entre os níveis séricos de marcadores de remodelação óssea e reguladores da via Wnt e a densidade mineral óssea também foi analisada. Foram avaliados 128 pacientes submetidos à artroplastia da anca, com idade superior a 40 anos, a partir de uma base de dados clínica. Regressão linear foi utilizada para avaliar as associações supramencionadas. Associações entre os estimadores foram realizadas para imputar valores ausentes na base de dados. A regressão *stepwise* foi usada, juntamente com o processo de eliminação *Backward* e bootstrapping foi usado para externamente validar os modelos. Além dos biomarcadores ósseos, quatro variáveis foram incluídas nos modelos: sexo, artrite reumatoide, uso de corticoide e osteoporose secundária.

Foram encontradas correlações positivas entre os níveis séricos de reguladores da via Wnt (P1NP, SOST e Dkk1) e a densidade mineral óssea. No que diz respeito à expressão genética dos biomarcadores ósseos, Dkk2 e sFRP1 foram negativamente associados à densidade mineral óssea, enquanto Lrp6 e WIF1 foram positivamente correlacionados. Estes resultados demonstram que uma regulação positiva dos reguladores da via Wnt, mais especificamente alguns dos inibidores da via Wnt, está associada a baixa massa óssea. O reduzido número de doentes é uma limitação e estudos futuros necessitam de ser realizados em populações maiores e com a inclusão de mais biomarcadores ósseos.

Esta dissertação foi feita no âmbito do projeto ARIBOS, financiado pela Sociedade Portuguesa de Reumatologia.

Palavras-Chave: Estimativa da densidade mineral óssea; remodelação óssea; Via Wnt; Biomarcadores ósseos; Osteoporose

Acronyms List

ALP Alkaline Phosphatase

BMD Bone Mineral Density

BMP Bone Morphogenetic Protein

BMU Basic Multicellular Unit

BRC Bone Remodelling Compartment

CBFA1 Core-binding Factor Alpha 1

CTX-I C-Terminal Telopeptide of Type I Collagen

DALY Disability-Adjusted Life-Year

DEXA Dual-Energy X-ray Absorptiometry

DKK1 Dickkopf factor 1

DKK2 Dickkopf factor 2

GUI Graphical User Interface

HRT Hormone Replacement Therapy

IGF Insulin-Like Growth Factor

IL Interleukin

IOF International Osteoporosis Foundation

LRP Low-density Lipoprotein receptor-related protein

M-CSF Macrophage-Colony Stimulating Factor

MSC Mesenchymal Stem Cell

OPG Osteoprotegerin

OPN Osteopontin

OSX Osterix

P1NP Procollagen Type I N-terminal Propeptide

PDGF Platelet-Derived Growth Factor

PGE_2 Prostaglandin E_2

PTH Parathyroid Hormone

PTHrP Parathyroid Hormone-related Protein

QCT Quantitative Computed Tomography

RANK Receptor Activator of Nuclear Factor *Kappa*

RANKL Receptor Activator of Nuclear Factor *Kappa* Ligand

SFRP1 Secreted Frizzled-related Protein 1

SEMA Semaphorin

TEM Transmission Electron Microscopy

TFM Total Fat Mass

TGF-β Transforming Growth Factor Beta

TLM Total Lean Mass

TNF Tumour Necrosis Factor

TRAP Tartrate-resistant Acid Phosphatase

VDR Vitamin D Receptor

WHO World Health Organization

WIF1 Wnt Inhibitory Factor 1

WNT Wingless/Integrated

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Chapter 1

Introduction

1.1 Osteoporosis and its Social and Economic Impact

Osteoporosis has become a major health concern worldwide, mainly due to its prevalence and socioeconomic consequences on society [1], [2], with currently more than 200 million people suffering from this disease [3]. Osteoporosis is characterized by low bone mass and structural degeneration, resulting in increased bone fragility and predisposition to fracture [4]. These fractures result in a major health issue in the elderly population, namely in terms of morbidity, mortality and costs to health care services [5], [6].

Osteoporosis occurs primarily due to normal ageing, where bone becomes naturally weaker over time. Bone mineral density starts to progressively decline at the fourth decade, whereas the fracture risk increases progressively [7]. However, it can also result from excessive bone loss during adulthood (e.g. menopause – in women) [8]. When women reach menopause, the steep decrease observed in oestrogen serum levels impairs the normal bone remodelling cycle, ultimately leading to a net loss of bone [9].

In 1994, the World Health Organization (WHO) proposed a simple method to classify osteoporosis, which was essentially based on the measurement of bone mass by dual-energy X-ray absorptiometry (DEXA) [10]. It classifies the subjects into four main classes according to the value of their bone mineral density (BMD), when compared with a young adult reference population (T-score, Table 1.1). Based on these diagnostic criteria, an estimated 27.6 million European residents had osteoporosis in 2010 [11].

Table 1.1 – Classification of osteoporosis according to WHO.

Category	T-score
Normal	T-score ≥ –1.0
Osteopenia	–2.5 < T-score < –1.0
Osteoporosis	T-score ≤ –2.5
Severe osteoporosis	T-score ≤ –2.5 with a fragility fracture

Nowadays, it is known that osteoporosis and the occurrence of osteoporotic fractures depend on several factors besides bone mass. These risk factors can be divided into major and minor risks, according to their impact in osteoporosis. Among the major risk factors are age and sex of the individual, prior fragility fractures, family history of hip fractures, corticoid therapy and hypogonadism. Minor risk factors include body mass index lower than 18.5 Kg/m^2, rheumatoid arthritis, hyperparathyroidism, frequent falls and toxic habits, such as excessive intake of alcohol and current smoking [6].

In 2008, the University of Sheffield, collaborating with WHO, developed a clinical algorithm, known as FRAX®, that expresses the 10-year probability of hip fracture and major osteoporotic fracture, like spine, forearm or shoulder fractures. This algorithm considers each of the individual patient models that integrate the clinical risk factors, as well as BMD at the femoral neck and provides a relatively reliable tool to categorize individuals according to the risk of fracture and, thus, prioritize pharmacological therapies [6], [12].

According to the International Osteoporosis Foundation, there are around 9 million fractures each year caused by osteoporosis, all over the world [13]. Europe and the Americas, by themselves, account for more than half of the share regarding the place of occurrence of these fractures and in a rapid aging population, consequence of declines in fertility rates and higher average life expectancy, these statistics will increase over the next decades [13]. The incidence of osteoporotic fractures increases exponentially with age and the existence of previous fragility fractures imposes a huge risk factor for subsequent fractures, underlining the importance of urgent intervention in patients to prevent further fractures and improve the quality of life (Fig. 1.1) [5].

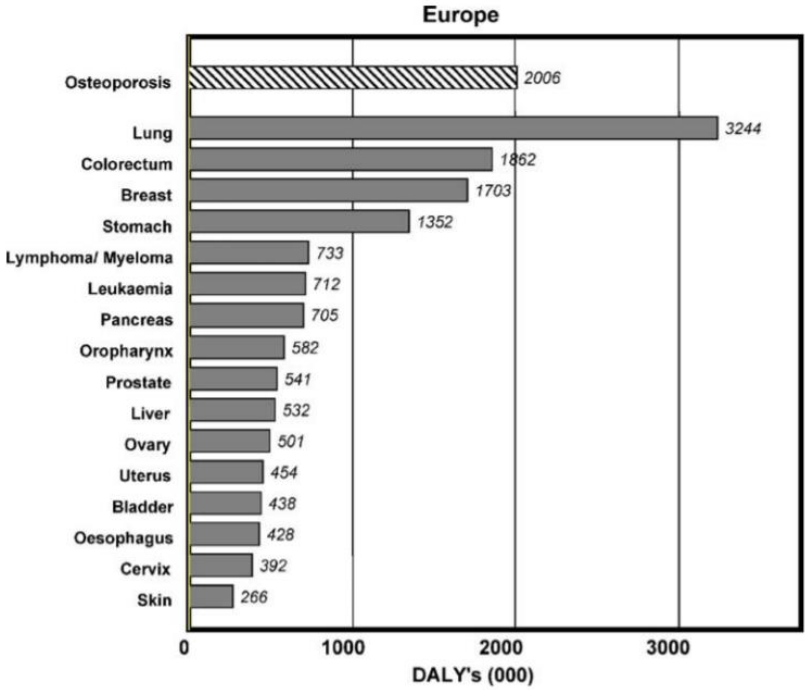


Figure 1.1 – Burden of osteoporosis in Europe and its comparison with other neoplastic disorders, measured by disability-adjusted life-years (DALYs) that are lost for each disease [14].

Concerning the economic costs, these include direct and indirect costs. The first ones are related to hospitalization and aftercare costs, whereas the second ones are meant for the impact on daily basis activities and, thus, quality of life. Together, these costs are a substantial economic burden on social and health care services [5]. To illustrate this reality, it is estimated that the direct costs of fractures derived from osteoporosis in Europe reach around €36 thousands of millions annually [15].

1.2 Standard Diagnostic Tools for the Assessment of Osteoporosis

Bone mineral density is one of the major predictors of osteoporosis and fracture risk, which makes it of utmost importance to accurately measure it. Since bone density cannot be measured by traditional X-rays, specialised techniques need to be used [16]. A variety of different techniques are available [10], namely peripheral DEXA (for forearm, finger and heel), single-energy X-ray absorptiometry (for heel or wrist), dual photon absorptiometry (for spine, hip or total body), single photon absorptiometry (for wrist), quantitative computed tomography (for spine or hip), peripheral quantitative computed tomography (for forearm) and quantitative ultrasound (for heel or finger). However, for the

purpose of this work, only the two currently most commonly used techniques will be addressed.

1.2.1 Dual-energy X-ray Absorptiometry

Nowadays, DEXA remains the gold standard diagnostic tool for osteoporosis, estimating bone mineral content (BMC) from the portions of the image identified as bone tissue and dividing it by the total projected area scanned [17]. Therefore, BMD measurements are obtained in grams per unit of area, which usually comes in cm^2 [17], [18].

Current DEXA equipments deliver much less radiation, protecting patients from excessive radiation exposure, which was one of the greatest disadvantages of this technology in its early developments [17]. Nevertheless, patients are still exposed to some extent of radiation, which implies a clear limitation as it presents a risk in patient health. Another feature allows DEXA to distinguish bone tissue from other tissues, such as fat and muscle, by using x-ray beams with different energies [18].

However, tissues are not homogenously distributed, which leads to measurement errors [19]. Moreover, correct patient positioning is of utmost importance, since slight changes in positioning result in a wide range of BMD values, often far from reality [20]. Other disadvantages include high associated costs, large-sized machines and relatively low reproducibility [21].

1.2.2 Quantitative Computed Tomography

Different diagnostic techniques have been used to quantify bone mass and bone loss as well. For instance, quantitative computed tomography (QCT) is utilized to obtain volumetric measurements of BMD [22]. These measurements represent a value closer to reality, as the information does not get partially lost during areal projection calculations [23]. In addition, it is possible to separate cortical from trabecular bone, a great advantage over DEXA [21], [22].

Although having significant advantages compared to DEXA, QCT comes with a few disadvantages as well, very similar to those observed in DEXA machines. Even being considered a more advanced technology, its high complexity requires well-trained specialists to procced with the scanning protocols [21]. High costs are also among the

disadvantages, together with the emission of higher radiation doses when used to measure hip BMD values [21].

Therefore, around 2008, the International Society for Clinical Densitometry (ISCD) established that QCT should be used to perform vertebral measurements, while hip measurements could be evaluated with DEXA or peripheral CT scanners (pQCT) [24].

1.3 Motivation and Objectives

Bone remodelling is a lifelong process, where bone tissue is constantly being removed and replaced by new bone tissue, commonly known as osteoclast-mediated bone resorption and osteoblast-mediated bone formation, respectively [25]. It plays a vital role in preserving and maintaining the skeleton healthy status during its lifetime, which includes bone replacement following any kind of injurie, such as micro-fractures, and responding to eventual body demands, depending on the mechanical load [25]. Changes in BMD occur naturally with advancing age but can also result from impaired bone remodelling activity due to possible existing abnormalities [26]. In impaired remodelling, abnormal changes in expression and release of various local and systemic factors often result in increased bone resorption activity, leading to decreased BMD and bone mass loss [25], [26].

Bone mineral density is a measure of bone mass per unit of volume (volumetric density) or per unit of area (areal density) and can be assessed through densitometric techniques [11]. BMD is one of the major predictors of osteoporosis and fracture risk, which makes it a key-factor in the diagnosis of osteoporosis. Early diagnosis of osteoporosis is essential for the identification of patients with a high risk of developing osteoporosis and fragility fractures, resulting in early and more efficient treatments. Additionally, the use of models capable of estimating BMD based on measured serum levels of bone biomarkers and/or genetic expression of bone biomarkers, could help to reduce both the need of *in vitro/in vivo* studies, which are extremely expensive, and the frequency of DEXA exams prescribed. In addition, these models may lead to vital clues regarding biomarkers that can be used as powerful markers for osteoporosis treatment decision.

Hence, the main objective of this dissertation is to **assess the association between bone gene expression and serum concentrations of markers of osteoblast and osteoclast differentiation and of Wnt pathway regulators with bone mineral density**. In order to achieve the proposed main objective, the following specific objectives were defined:

1) Estimate bone mineral density based on its major contributors;
2) Evaluate the association of serum levels of markers of osteoblast and osteoclast differentiation and of Wnt pathway regulators with bone mineral density;
3) Estimate the association between bone gene expression markers of osteoblast and osteoclast differentiation and of Wnt pathway regulators and bone mineral density.

This dissertation was conducted under the project ARIBOS, funded by the Portuguese Society of Rheumatology.

1.4 Thesis Outline

Apart from this chapter, which contemplates a brief introduction about osteoporosis, as well as the importance of this work and the motivation that propelled it, an extensive and detailed insight into the biology and physiology of bone is given in Chapter 2.

Chapter 2 is crucial, because it explains the complex yet brilliant architecture of bone, its mechanical properties and microenvironment, namely a description of the bone cells, the major determinants of bone mineral density and the markers of osteoblast and osteoclast differentiation, with emphasis in the Wingless/Integrated signalling pathway.

Chapter 3 provides a description of the methodology used. More specifically, it contains information about the study design, source of data, study variables and the statistical data analysis performed.

Chapter 4 presents the exploratory analysis of the data and the main results obtained, as well as a glimpse into the developed graphical user interface.

Chapter 5 discusses the results obtained and compares them with the literature, as well as exploiting some of the limitations of this work.

Chapter 6 resumes the main conclusions drawn from this work and discusses future perspectives.

Chapter 2

Literature Review

In this chapter, the theoretical concepts that are important to understand the underlying complexity of bone and all its biophysiological processes are discussed in detail. The major determinants of bone mineral density are addressed and explained, together with numerous bone resorption and bone formation biomarkers. Finally, the Wingless/Integrated (Wnt) signalling pathway is explained, as well as the bone biomarkers that are part of it.

2.1 Bone Physiology and Biology

The vertebrate skeleton is made of different tissues, each one with different properties and functions, that provide support, structure and protection for the body. It is mainly composed by bone, but it also includes cartilage, tendons and ligaments. On the other hand, bone is a unique kind of connective tissue with a characteristic that distinguishes it apart from the rest of the connective tissues, which is its mineralized composition. As an organ, bone is made up of cartilaginous joints, calcified cartilage of the growth plate (exclusively during skeleton growth), marrow space and the mineralized structures of cortical (compact bone) and trabecular (cancellous bone) bone (Fig. 2.1) [25], [27], [28].

As a tissue, bone is a combination of the mineralized and non-mineralized matrixes (osteoid) and the cellular components, namely the osteoblasts, responsible for bone formation, the osteoclasts, responsible for bone resorption, and the entrapped osteoblasts into the mineralized matrix that later differentiate into osteocytes. The communication between these cells and the role they play in bone remodelling processes will be further discussed below [25], [27], [28].

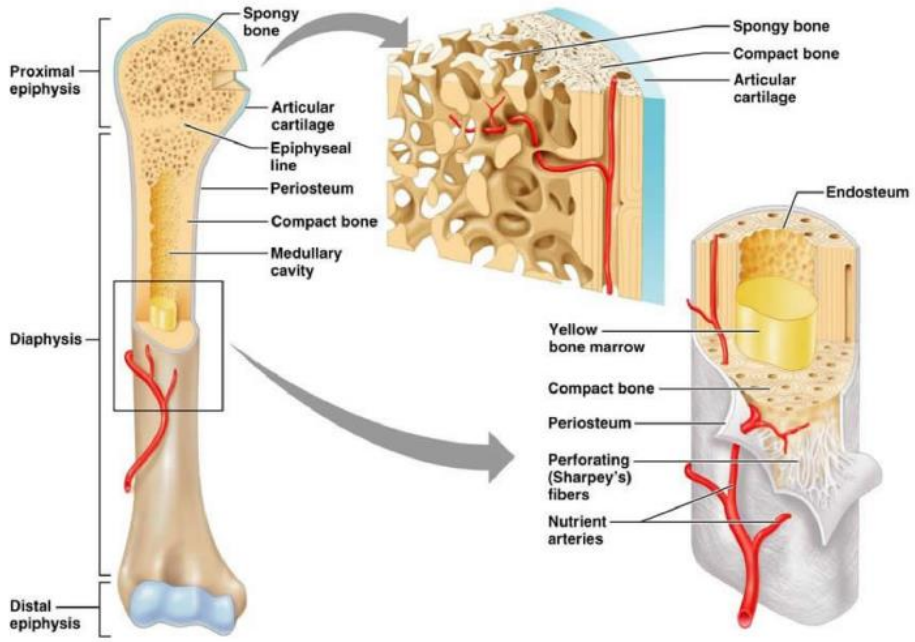


Figure 2.1 – Constitution of long bones. At a macroscopic scale, long bones are composed by articular cartilage, the inner layer and porous trabecular bone, the outer layer and denser cortical bone and the marrow cavity. On its surface, long bones are covered by a thin fibro-cellular highly vascularized and innervated membrane, the periosteum [25], [29].

Cortical bone is a dense, compact, low metabolism type of bone and forms the hard-outer layer, called cortex, giving its smooth, white and solid appearance. It facilitates the execution of the main bone functions, which includes supporting the body, protecting vital organs, providing levers for movement, and storing chemical ions. It consists of multiple microscopic columns (osteon), composed by multiple layers of osteoblasts and osteocytes around a central canal called the Haversian canal. The Volkmann canal is responsible for addressing a connection between the Haversian canals [25], [27], [28]. On the other hand, trabecular bone is a cancellous, high metabolism type of bone with a high level of porosity. It forms the internal tissue of skeletal bone and is an open cell porous network. Trabecular bone has a higher surface-area-to-volume ratio than cortical bone and it makes the bone softer and weaker, but at the same time more flexible. This type of bone is highly vascular and frequently contains red bone marrow, where blood cells are produced. Thin formations of osteoblast, covered in endosteum, create an irregular network of spaces, the trabeculae, with its functional unit, the trabecula [25], [27], [28]. Figure 2.2 shows the structure and the individual components of cortical bone, while

figure 2.3 demonstrates the arrangement of the components in trabecular bone, together with a transverse section of a trabecula [30].

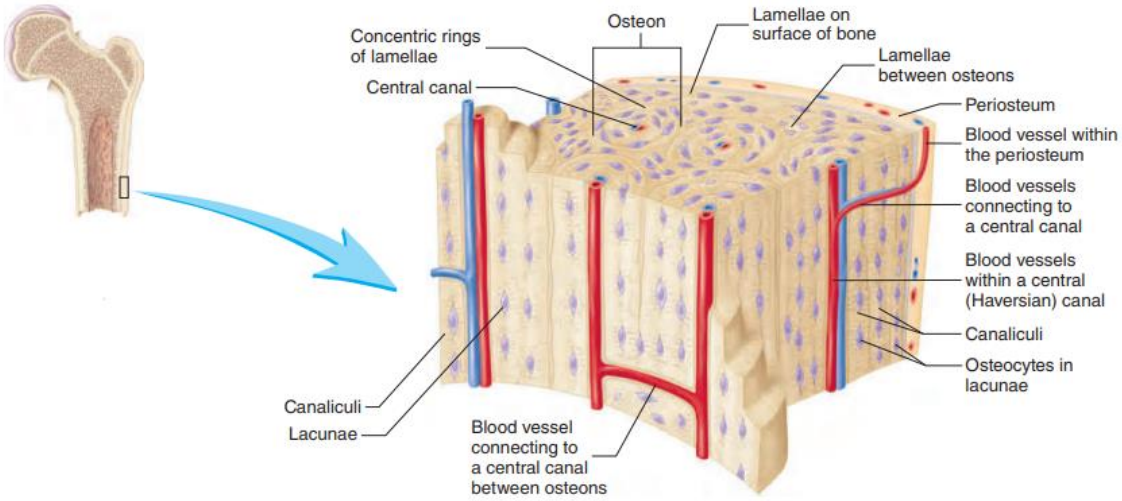


Figure 2.2 – Structure and components of cortical bone. Adapted from [30].

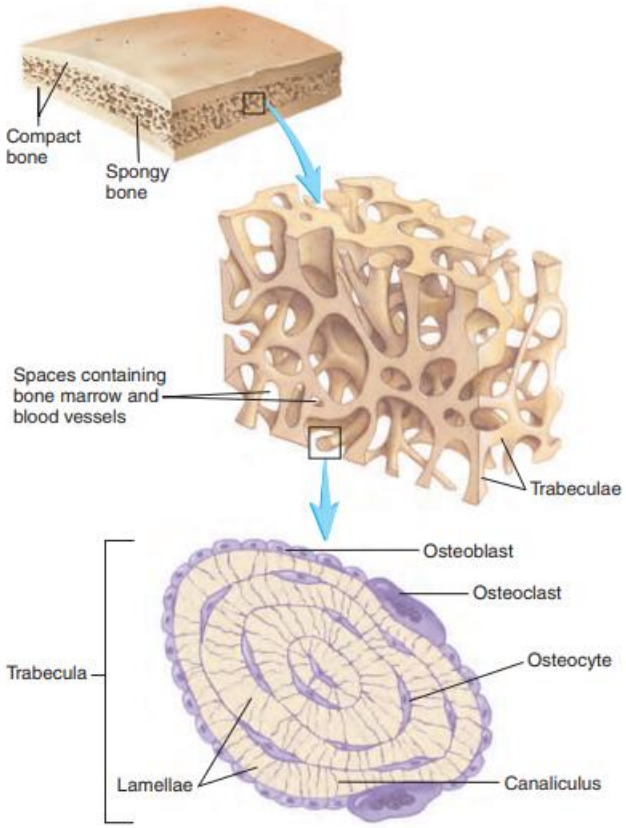


Figure 2.3 – Structure and components of trabecular bone. Adapted from [30].

2.1.1 Hierarchical Bone Structure

Bone is organized in a variety of material arrangement structures, to achieve its mechanical, biological and chemical functions (Fig. 2.4). To understand the bone mechanical properties, it is important to analyse the combined mechanical properties of its individual components, as well as their relationship at the various levels of hierarchical structural organization [25], [27], [28].

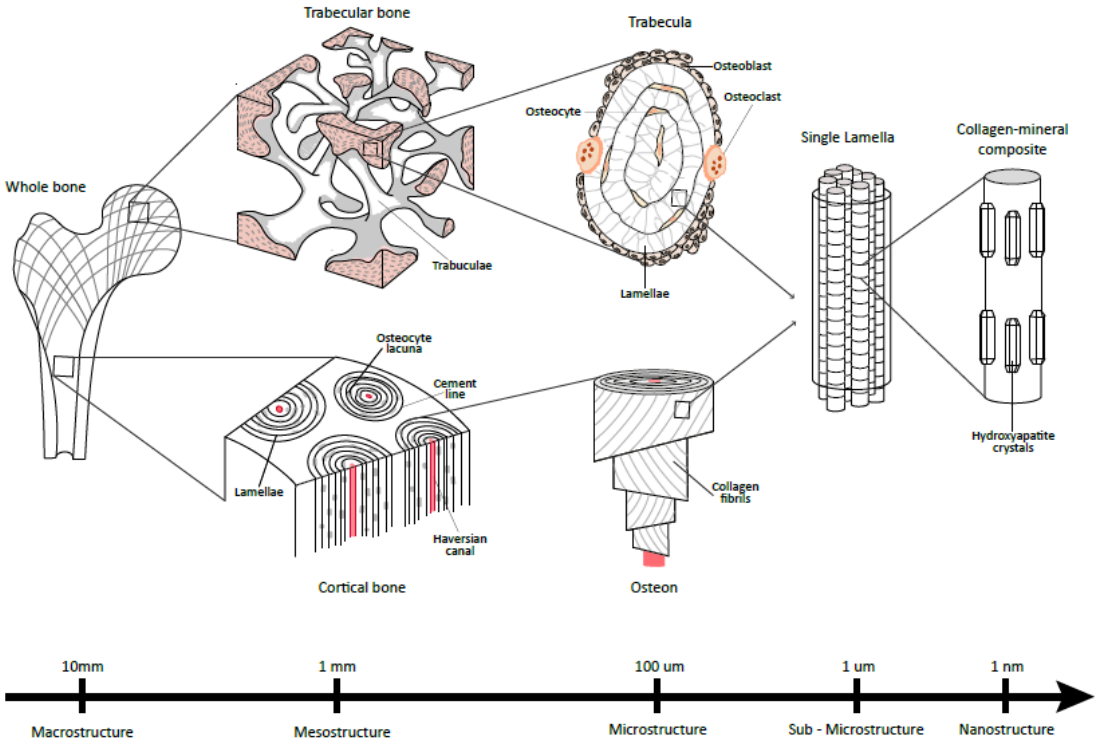


Figure 2.4 – Hierarchical bone structure. At the macrostructure level, bone is the combination between trabecular and cortical bone. At the microstructure level, the Haversian systems, which are a series of canals to accommodate blood vessels, are surrounded by layers of concentric lamellae. The connection between these systems is assured by the transversal Volkmann canals. At the nanostructure level, bone is made of two phases: the organic phase is composed by the collagen type I organic matrix, whereas the mineral phase is composed by carbonate-substituted hydroxyapatite embedded in the organic matrix. Adapted from [31].

At a macrostructure point of view, bone is composed by its inner and porous trabecular bone with the marrow cavity, involved by a dense cortical bone, acting as a protective shield. At this level, bone mechanical properties are studied through classic bending

tests, to analyse bone stiffness, energy storage, ductility and fragility, among others [25], [27], [28].

At a microstructure point of view, cortical bone has numerous concentric lamellae layers that surround a central canal carrying a blood vessel, commonly known as the Haversian systems (osteons). These systems form an interconnected grid through transversal channels, designated as Volkmann canals. Trabecular bone has cellular foam-like structure made of an interconnected network of rods and plates forming the bone trabeculae. Osteons and bone trabeculae have osteocyte lacunae, connecting to each other and to vessels through canaliculi [25], [27], [28].

Finally, at a nanostructure point of view, transmission electron microscopy (TEM) allowed the concept of bone as a heterogeneous and anisotropic material that comprises two phases: the organic phase, formed by the organic matrix, is mainly composed by collagen type I and the mineral phase is essentially composed of inorganic particles of carbonate-substituted hydroxyapatite embedded in the organic matrix [25], [27], [28].

2.1.2 Bone Mechanical Properties

Since bone is a highly hierarchically structured tissue, its strength arrives from the complex, yet harmonized relationship between the macroscopic tissues (cortical and trabecular bone) and the different structural and material properties [32]. Bone can adapt itself to mechanical load [33]. However, its adaptive response varies, depending on the strain conditions (frequency, distribution, amplitude, among others). The mechanical behaviour of bone reflects its intrinsic properties, and these can be determined through standardized mechanical tests [34].

2.1.2.1 Stress-Strain

When external forces (stress) are applied to bone, they are distributed over its cross-sectional area and induce structural deformations (strain). This results in the generation of an internal resistance to the applied force. While strain is a measure of linear deformation, expressed in percentage (%) of change from the original dimensions or angular configuration of the structure, stress is a measure of load per unit of area, expressed in Newton per square metre (N/m^2) or Pascal (Pa) [32]. Considering the stress-strain curve, bone can either exhibit elastic behaviours or plastic behaviours, depending on its yield

point. There is a linear relationship between the stress applied and the resultant deformation, expressed by the modulus of Young (E). It is obtained by determining the slope of the linear portion of the curve, dividing the stress by the strain at any point in that region (Fig. 2.5).

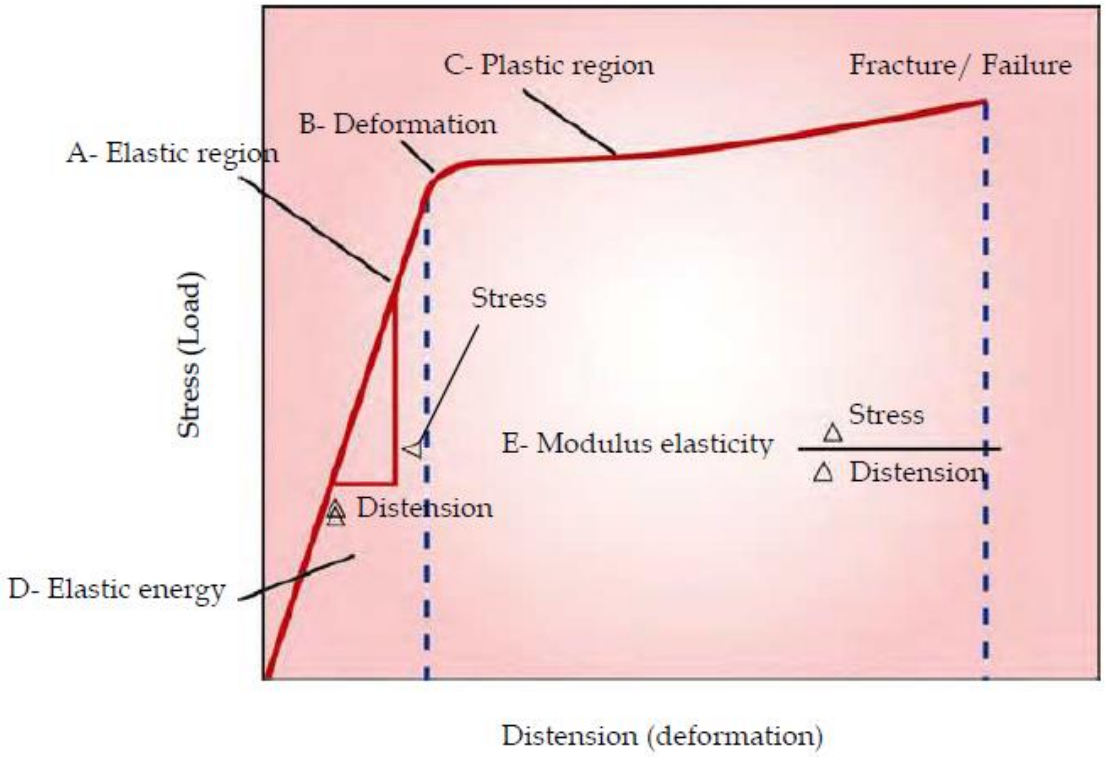


Figure 2.5 – Representation of a stress-distension curve. The elastic region (A) represents a region where bone has the capability to restore its initial form. From that point, permanent deformation occurs (B), entering in a new region, called the plastic region (C). Eventually, bone reaches a point where fracture will occur. The slope of the curve, in the elastic region, represents the modulus of Young (E) and the material strength is determined by the area under the total curve (D) [34].

When the magnitude of the strain remains under the yield point, designated as the elastic region, bone elastically deforms by storing energy and later returns to its initial state by releasing the absorbed energy. In contrast, when the magnitude of the strain exceeds the yield point, bone enters a so-called plastic region, in which bone loses its ability to return to its initial state, resulting in permanent damages.

2.1.2.2 Mechanical Behaviour

In order to withstand a variety of loading types, bone has distinctive responses to each one of them, due to its unique anisotropic architecture. Bone has adapted itself to accommodate the load, which resulted in bone strength and rigidity being typically greater in the direction where load is most commonly applied [32], [34]. Stress can be categorized into five types: compression, tension, shear, torsion and bending (Fig. 2.6).

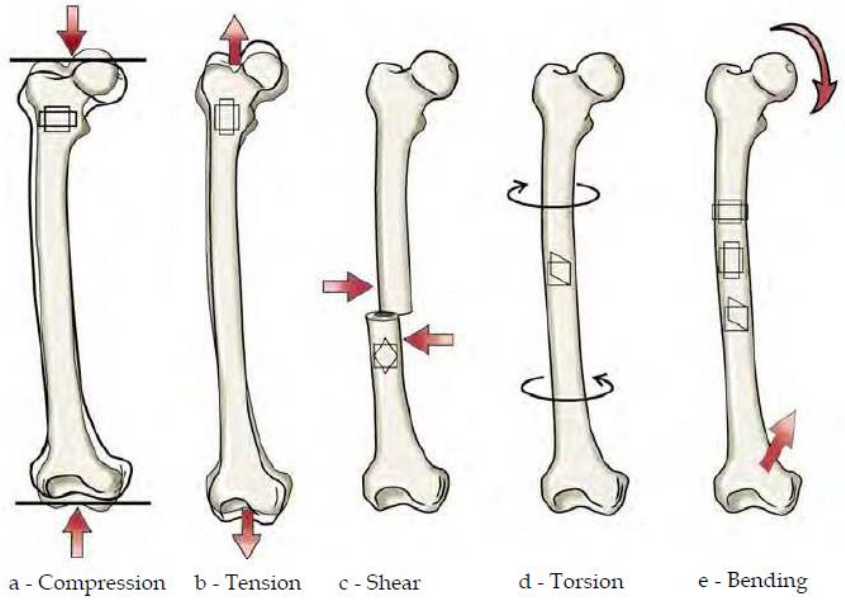


Figure 2.6 – Schematic illustration of different types of external forces that can be applied to bone [34].

Cortical bone, where osteons are oriented in a longitudinal direction, can better handle compressive strains than tensile strains, but does not withstand shear rates very well due to its high stiffness [35]. Trabecular bone has a lower Young modulus than cortical bone, because it has a greater porosity degree [36], and although less stiff, it can withstand greater strains.

Considering the material contribution, the organic and inorganic components present in bone influence the mechanical behaviour as well [37]. The degree of mineralization (together with its quality) and porosity are the two major factors that determine the quality of bone and how it will respond to load [32]. When bone becomes overly stiff and brittle, due to its high levels of mineralization and crystallinity, the formation of microfractures can emerge at lower levels of deformation [32]. Conversely, if the levels of mineralization and crystallinity are too low, bone becomes more fragile and weaker, leading to the

appearance of microdamage at lower levels of deformation as well [32]. On the other hand, the degree of porosity strongly influences bone mass and density [37]. The rapid age-related deterioration on the cortical and trabecular structures increases bone porosity, compromising bone integrity and leading to a successive bone loss over time [32]. Therefore, an optimal balance between the quality of both organic and inorganic components, influenced by the degrees of mineralization, crystallinity and porosity, is vital to maintain the best standards of bone response to load [32], [33], [37].

2.1.3 Bone Cells

2.1.3.1 Osteoblasts

Osteoblasts are the cells responsible for creating new bone tissue and emerge from mesenchymal stem cells (MSCs). They synthesize osteoid, which is a non-mineralized organic extracellular matrix [25], [27], [28].

After participating in bone formation processes, osteoblasts undergo one of three possible outcomes: 1) they go through apoptosis (programmed cell death), 2) differentiate into osteocytes if they become trapped into the mineralized bone matrix, or 3) become dormant lining cells, participating in calcium-exchange activities and waiting to be reactivated to engage in bone formation [25], [27], [28].

The osteoblast-lineage cells give rise to osteoblast progenitor cells (pre-osteoblasts), that later differentiate into mature osteoblasts. The process of osteoblast differentiation is stimulated by the presence of specific chemical signalling cytokines, which include bone morphogenetic proteins (BMPs), parathyroid hormone (PTH), transforming growth factor beta (TGF-β) and a wingless/integrated (Wnt) pathway. Osteoblasts themselves are capable of producing growth factors as well, such as insulin-like growth factor I and II, TGF-β and BMPs, that are stored in the organic matrix and play a vital role in osteoblast differentiation and normal functioning. The osteoblast-lineage maturing process is called osteoblastogenesis (Fig. 2.7) [25].

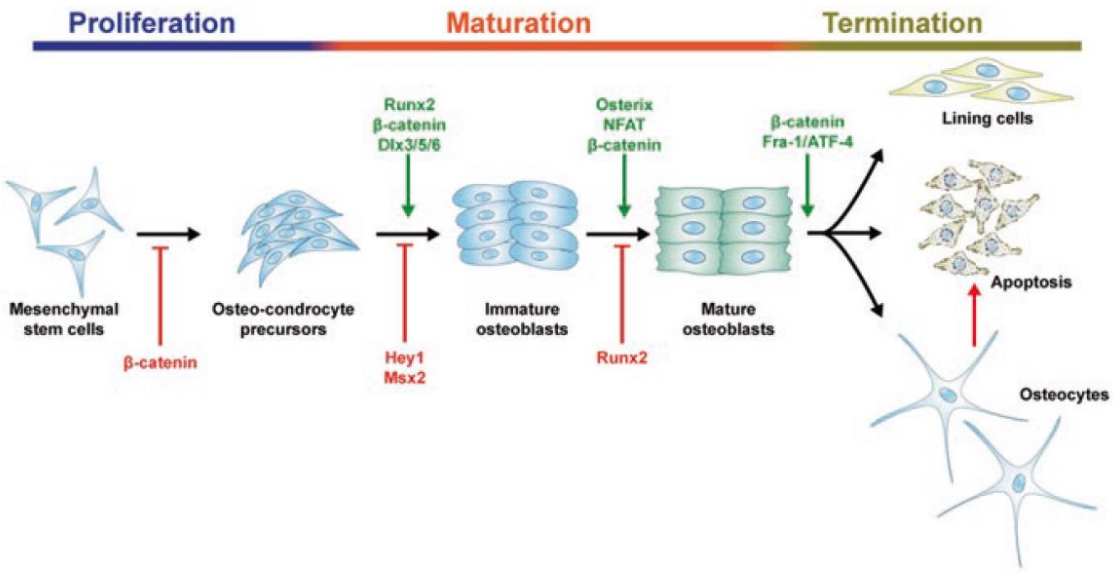


Figure 2.7 – Osteoblastogenesis. Active osteoblasts arise from a sequential differentiation of mesenchymal stem cells and participate in bone formation. Upon termination of bone formation, osteoblasts can become lining cells on the bone surface, die by apoptosis, or differentiate to osteocytes [25].

2.1.3.2 Osteocytes

Osteocytes are the differentiation products of osteoblasts that become trapped in the mineralized bone matrix. Osteocytes are uniformly distributed throughout the mineralized bone matrix and connect between each other or to other cells through dendrite-like processes contained in fluid-filled canals (canaliculi), which radiate toward the surface and the blood supply [25], [27], [28].

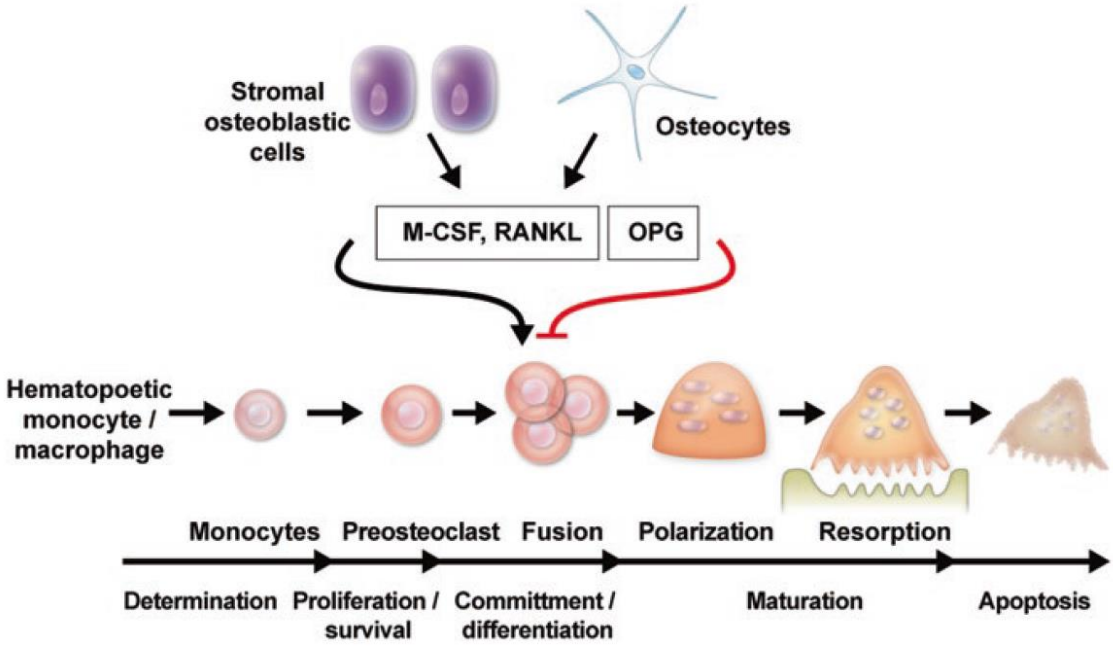
Their strategical location in the bone matrix allows them to detect local changes in mechanical stimuli, respond to changes in concentrations of circulating factors, such as hormones and ions, and to amplify these signals, leading to a coordinated adaptive response of the skeleton to environmental changes, which include adaptations in bone architecture, through bone modelling. Hence, osteocytes are extremely important cells in adapting bone to variations in mechanical loading and maintaining skeletal health [25], [27], [28].

Sclerostin is an important marker for mature osteocytes, encoded by the SOST protein and acts as a negative key regulator of bone mass by counteracting the canonical Wnt signalling cascade, which is crucial to bone homeostasis. It affects bone formation directly and bone degradation indirectly, and its withdrawal results in increased bone mass

[38]. Osteocytes are long-lived cells, but like all cells, they also undergo apoptosis. A decrease in the osteocyte population is associated with bone fragility induced by oestrogen deficiency, glucocorticoid excess and lack of mechanical load [25], [27], [28].

2.1.3.3 Osteoclasts

Osteoclasts are the cells responsible for bone resorption. They are members of the monocyte-macrophage family derived from bone marrow macrophages. The osteoclast differentiation process, known as osteoclastogenesis (Fig. 2.8), depends on two cytokines, namely, macrophage-colony stimulating factor (M-CSF or CSF-1) and receptor activator of nuclear factor- κB ligand (RANKL), which are produced by marrow stromal cells and



osteoblasts [39].

Figure 2.8 – Osteoclastogenesis. Osteoblasts and osteocytes secret RANKL and M-CSF, among other cytokines, that control osteoclast differentiation. Osteoprotegerin (OPG), which is also secreted by osteoblasts and osteocytes, acts as an inhibitor for osteoclast differentiation, by binding to RANKL [25].

RANKL is a member of the tumour necrosis factor (TNF) family and is the key osteoclastogenic cytokine required for the differentiation of precursor cells. RANKL, produced by osteoblast and osteocytes, binds to the RANK receptor, which is expressed in osteoclasts.

Osteoclastogenesis is stimulated by PTH, PTH-related protein (PTHrP), prostaglandin E_2 (PGE_2) and interleukin 1 (IL-1), which act on osteoblast cells to upregulate RANKL expression [25], [27], [28].

In contrast, OPG, a form of the TNF receptor, which is also expressed by osteoblast and osteocytes and is upregulated by oestrogen, BMP and TGF-β, acts as a decoy receptor for RANKL to downregulate osteoclastogenesis. Therefore, the ratio between RANKL and OPG in osteoblast cells controls differentiation and activation of osteoclasts [40], [41].

2.1.4 Bone Remodelling

As a dynamic organ, bone undergoes a continual and coordinated self-regeneration process throughout life, changing its internal structure in a process called remodelling [42]. Bone remodelling requires osteoclast-mediated bone resorption and osteoblast-mediated bone formation and it is achieved by the basic multicellular unit (BMU) [43]. In cortical bone, an osteonal structure is formed, which represents concentric layers of bone encircling a Haversian canal. Conversely, when remodelling occurs on trabecular bone, the resulting structure is a hemiosteon.

The remodelling cycle is divided into five stages: activation, resorption, reversal, formation and quiescence [25], [43], [44]. The activation phase begins with the lining cells digesting the layer of unmineralized matrix, thus exposing the bone surface. Osteoclast precursors are recruited to the bone surface, differentiating into functioning osteoclasts. Afterwards, the bone lining cells retract to expose the mineralized matrix, allowing osteoclasts to attach and initiate bone resorption.

During bone resorption, osteoclasts first dissolve the mineralized bone matrix and later degrade the organic matrix. After completion, they undergo apoptosis. The reversal phase, which comes after the resorption phase, is a time window in which bone resorption terminates and bone formation begins. Here, specialized cells deposit a thin layer of collagen rich in osteopontin (OPN) that provides fibre matrix bonding, enhancing bone resistance to fracture.

During the formation phase, osteoblasts deposit the osteoid, which consists primarily of type I collagen fibres and serves as a template for the mineralization. At last, the quiescence phase is characterized by a latency state, in which bone rests until a new cycle begins.

While bone is being formed, osteoblasts deposit growth factors in the mineralizing matrix, such as TGF-β, platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs) and bone morphogenetic protein 2 (BMP-2). These growth factors can be later released following bone degradation by osteoclasts and participate in the stimulation, recruitment, migration, and differentiation of osteoblast progenitors [45].

Hauge [46] suggested a model for cancellous bone remodelling, in which a closed shell-form compartment is formed to promote the occurrence of the process (Fig. 2.9). Since the bone remodelling compartment (BRC) is sealed, it may contribute to keep the local chemical signals at concentrations sufficiently high to promote the fully completion of the bone remodelling cycle. It has gained support over the years, because it may help to explain how bone resorption and bone formation are linked, even though they take place at different time windows during the cycle.

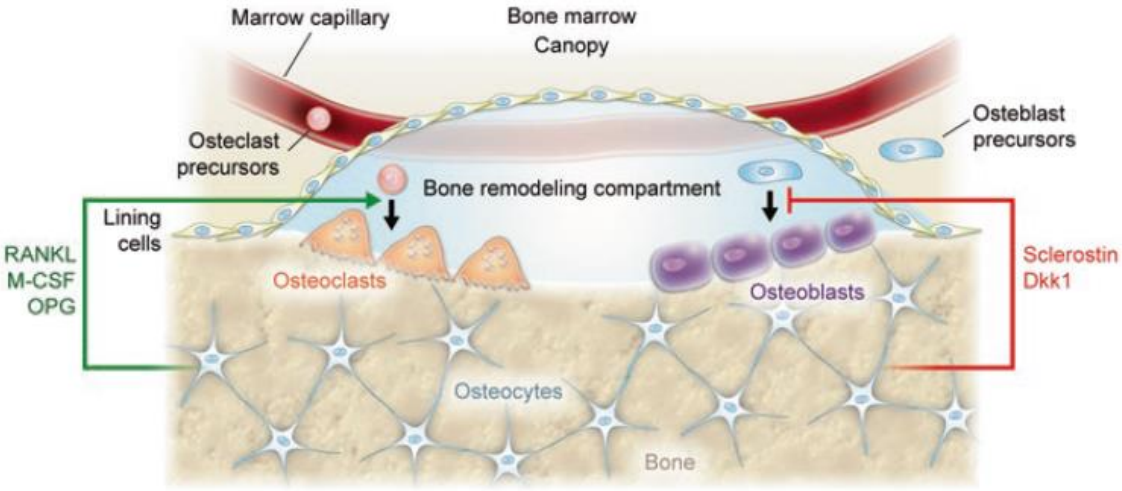


Figure 2.9 – Bone remodelling compartment. For the bone remodelling process to occur, osteocytes send a signal to the neighbouring bone lining cells, which retract and expose the bone surface. Osteoclast precursors are recruited and differentiate into mature osteoclasts to initiate bone consumption. As the degradation of the bone matrix progresses, the embedded growth factors are released, thereby promoting the recruitment of osteoblast precursors and their differentiation into mature osteoblasts. After bone resorption is complete, osteoblasts initiate bone formation, creating new bone matrix and refiling the lacuna. The green arrow indicates stimulation, whereas the red arrow indicates inhibition [25].

2.2 Major Determinants of Bone Mineral Density

Various studies have shown that body weight, body composition and body fat distribution, dietary calcium and vitamin D intake and physical activity represent major drivers of bone mineral density in adults and elderly populations [47]–[54].

2.2.1 Body Weight

Macdonald *et al.* [55] demonstrated that body weight accounted for 2.6% of bone loss at the femoral neck and 8.4% at the lumbar spine in postmenopausal women, who were not under any type of hormonal replacement therapy (HRT) treatment. These results showed that a variation in body weight was, indeed, a significant predictor of BMD and were in agreement with the findings of Marcel *et al.* [50], which reported as well that body weight was a major determinant of BMD.

Finkelstein *et al.* [56] also concluded that body weight had a strong impact on BMD loss after menopause. Meanwhile, numerous studies have reported that women with higher body mass index and weight were negatively correlated with the rates of bone loss, meaning that thinner women are most likely to develop osteoporosis, due to their higher bone loss rates [57]–[60].

Exactly why women with higher body weight have slower bone loss rates is unclear, but a possible explanation to this phenomenon might be related to increased productions of oestrogens by adipose tissue [56].

2.2.2 Body Composition and Body Fat Distribution

Body composition is considered an important factor, regulating bone mass in postmenopausal women [49]. A significant number of studies discovered that fat mass was positively associated with BMD [51], [55], [61]–[64] and just like Finkelstein *et al.* [56], these authors attributed this discovery to possible increased productions of oestrogens by the adipose tissue.

Other researchers have suggested that women with higher body fat percentages naturally impose higher loads to the bone, leading to increased mechanical stimulus and, consequently, increased BMD [65]–[67].

Miliken and his co-workers [47] demonstrated that the variations in soft tissue composition better explained the changes in BMD, compared with body weight, exercise frequency or calcium intake. Gnudi *et al.* [68] studied the relationship between total fat mass (TFM) and total lean mass (TLM) with BMD and BMC. They concluded that both TFM and TLM were significantly correlated with BMD and BMC in osteoporotic and non-osteoporotic postmenopausal women.

2.2.3 Dietary Calcium and Vitamin D Intake

It has been established that nutritional factors have an impact on bone mineral density and age-related bone loss [69]. Calcium plays a vital role in skeletal health and its impact on bone mass has been extensively studied [70]. On the other hand, vitamin D deficiency is well known among elderly people, as a consequence of low sunlight exposure and low dietary vitamin D intake [71]. The metabolic process of converting 25(OH) Vitamin D into $1.25(OH)_2$ Vitamin D decreases with age, which results in lower absorptions of calcium in the intestine [72], [73]. In turn, this leads to production of PTH and stimulation of its catabolic effect, increasing bone resorption [74], [75].

Several studies have shown that regular calcium intake is positively correlated with BMD and BMC [48], [76]–[79]. Tai *et al.* [80] studied whereas higher calcium intakes affected BMD and concluded that it led to small increases in BMD. Chevalley *et al.* [81] demonstrated that oral intake of calcium supplements successfully prevented decreases in femoral BMD and lowered vertebral fracture rate in vitamin-D-replete elderly patients. Grados *et al.* [71] reported significantly increases in BMD in older women who had vitamin D deficiency and were given supplementary calcium and vitamin D. Sadat-Ali and his colleagues [82] investigated the association between 25(OH) Vitamin D serum levels and BMD among Saudi individuals. They discovered that decreased serum levels of 25(OH) Vitamin D were associated with low bone mass and increased levels of PTH.

2.2.4 Physical Activity

During menopause and the following years, women experience significant reductions in muscular strength and bone mass [49]. Studies have shown that regular physical activity in postmenopausal women helps to maintain or even slightly increase BMD [83]–[89].

Chien *et al.* [90] demonstrated that aerobic exercises practiced on a regular basis attenuated bone loss.

On the other hand, Douchi *et al.* [87] reported that increases in body fat percentage due to sedentarism were related to increases in BMD in women. Hence, following an active lifestyle positively affects BMD maintenance and reduces bone loss [49].

Nevertheless, some studies found that physical activity levels had no impact on BMD [85], [91]. Thus, healthy active lifestyles should be adopted to prevent excessive bone loss, stimulating bone formation and helping to maintain BMD.

2.3 Bone Turnover Biomarkers

Bone biomarkers are chemical substances produced during the bone remodelling process that can provide valuable information concerning bone metabolism [92]. These can be divided into three categories: bone resorption, bone formation and bone turnover biomarkers. The latest are used to evaluate to what extent are bone resorption or bone formation being inhibited [92].

2.3.1 Indicators of Bone Resorption

Bone resorption markers are products that arise from the activity of osteoclasts during bone matrix degradation or cytokines that promote osteoclast differentiation and maturation [92]. Several indicators of bone resorption have already been proposed, but for the purpose of this work, we will only focus on those that were measured in the patients present in the database.

2.3.1.1 C-terminal telopeptide of type I collagen

Telopeptides are non-helical sequences of collagen that can be found at each end of the molecule [93]. C-terminal telopeptide of type I collagen, better known as CTX-I, is a degradation product from type I collagen (constituent of bone matrix) released during the activity of the enzyme cathepsin K [94]. CTX-I has been considered as a strong reference marker for bone resorption by the International Osteoporosis Foundation (IOF) [94].

2.3.1.2 Receptor activator of nuclear factor-κB ligand

Receptor activator of nuclear factor-KB ligand (RANKL) is a protein of the tumour necrosis factor (TNF) family [95], [96]. Produced by osteoblast-lineage cells, its function lies in the regulation of the differentiation, proliferation and maturation processes of osteoclasts, by binding to its receptor RANK [97].

Previous studies have shown that elevated RANKL serum levels in mice (by inhibiting its decoy receptor, OPG) lead to increased osteoclast activity and high rates of bone resorption; on the other hand, low-expression of RANKL resulted in a decrease in osteoclast activity and elevated bone mass [98], [99].

2.3.1.3 Tartrate-resistant acid phosphatase

Tartrate-resistant acid phosphatase (TRAP) is an inactive synthesized proenzyme. Its activated form, tartrate-resistant acid phosphatase isoform 5b (TRAP 5b), is established as an indicator of osteoclast activity and bone resorption [100], [101]. TRAP 5b, usually secreted by osteoclasts, is responsible for dephosphorylating proteins embedded in the bone matrix. In addition, it has the capability of producing reactive oxygen species necessary for bone matrix degradation [92], [101].

A study conducted by Halleen and his co-workers [102], demonstrated that serum levels of TRAP 5b were higher in osteoporotic patients and, consequently, negatively associated with BMD.

2.3.2 Indicators of Bone Formation

Concerning the indicators of bone formation, these are cytokines that arise from the activity of osteoblasts during bone formation or promote osteoblast differentiation, proliferation and maturation [92]. Numerous bone formation markers have been proposed as well. However, as mentioned previously, focus will be given only to those that were measured in the patients present in the database.

2.3.2.1 Alkaline phosphatase (bone isoenzyme)

Alkaline phosphatase (ALP) is a metalloenzyme and a vital piece in hard mineralized tissue formation [103]. Several studies have reinforced this knowledge, demonstrating that ALP leads, indeed, to tissue mineralization [104]–[109]. ALP is expressed in the early phases of tissue development, when other essential cytokines, such as osteocalcin, are still down-regulated [103]. ALP has, therefore, become a reference marker for evaluating the maturity of mineralized tissue and a good indicator of bone formation [103].

2.3.2.2 Osteocalcin

Osteocalcin is a protein secreted mostly by osteoblasts during bone formation activity [110]. Osteocalcin is often used as a biomarker of bone formation and is believed to have a regulatory function in bone tissue mineralization [110].

van de Loo *et al.* [111] studied the effect of osteocalcin on the precipitation of insoluble salts and demonstrated that osteocalcin played an inhibitory role on the precipitation of calcium salts. On the other hand, Ducy *et al.* [112] verified that mice with null expression of osteocalcin developed more bone mass and had higher bone formation rates compared to the control group. Sudhir *et al.* [113] concluded that, in his study of the diagnostic potential of serum osteocalcin levels as an indicator of primary osteoporosis in women, osteocalcin levels were negatively correlated with BMD measurements. These studies suggest that osteocalcin plays a vital role in inhibiting bone mineralization and can be used as a reference biomarker of bone formation.

Nevertheless, Murshed *et al.* [114] studied mice with elevated expression of osteocalcin and observed that bone mineralization exhibited, approximately, a normal status. Thus, further studies need to be conducted to assess the exact role of osteocalcin within the bone matrix environment.

To our knowledge, it can be used as a biomarker for osteoblastic bone formation, especially for women, since osteoporotic women present higher osteocalcin serum levels that non-osteoporotic women [113].

2.3.2.3 Core-binding factor alpha 1

Hormones and growth factors are required to activate osteoblast-specific signalling proteins and transcription factors and consequently stimulate osteoblast differentiation [115].

Core-binding factor alpha 1 (cbfa1), also known as runt-related gene 2 (Runx2), is one of the most important transcription factors for osteoblastic differentiation and osteogenesis [115]–[117]. It is responsible for regulating the expression of osteocalcin in fully mature osteoblasts [116], [118], ultimately playing an essential role in bone formation.

Komori *et al.* [119] performed a study on mice to evaluate the importance of cbfa1 in osteogenesis. They discovered that the deletion of this transcription factor resulted in a complete lack of ossification due to the maturational arrest of osteoblasts, proving that cbfa1 is a key regulator of osteoblast differentiation.

2.3.2.4 Procollagen type 1 N-terminal Propeptide

N-terminal propeptide of type I procollagen (P1NP) is a product derived from the cleavage of type I procollagen (Fig. 2.10) during the assemble process into fibrils [120]. Serum levels of P1NP are proportional to the amount of collagen embodied into the bone matrix, which results in a significant association with bone formation rates [120]. Therefore, P1NP is considered to be a standard reference biomarker for bone formation, as it reflects the activity of osteoblasts and bone formation and collagen deposition.

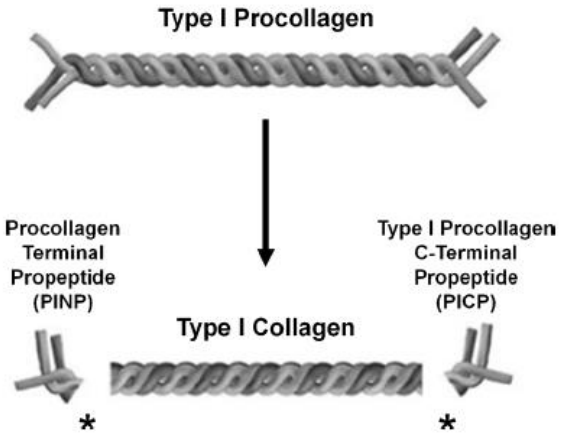


Figure 2.10 – The formation process of type I collagen, through the cleavage of the two terminals of type I procollagen. One terminal corresponds to the procollagen N-terminal propeptide (P1NP), while the other one corresponds to the procollagen C-terminal propeptide (P1CP) [121].

Elma *et al.* [122] studied the relationship between P1NP and BMD and discovered that serum levels of P1NP were significantly higher in osteoporotic women, compared to non-osteoporotic women.

2.3.2.5 Osterix

Osterix (Osx) is an osteoblast-specific transcription factor, vital for osteoblast differentiation and bone formation [123]–[125]. Nakashima and his co-workers [126] revealed, for the first time, that mice with knockout Osx did not develop any form of bone formation activity, although having normal cartilage development.

On the other hand, studies have shown that upregulating Osx resulted in expression of osteocalcin in osteoblasts and collagen type I [127]. However, recent studies have demonstrated that Osx also plays a role in inhibiting osteoblast proliferation, by binding to and activating Dkk1 (Fig. 2.11), thereby leading to an inhibitory role of the Wnt signalling pathway [123].

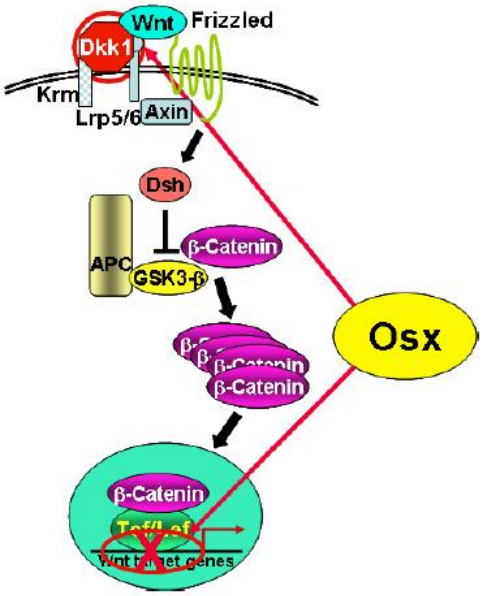


Figure 2.11 – Inhibitory mechanisms of osterix in the Wnt pathway. Osterix activates Dkk1, which is a natural Wnt inhibitor and inhibits the activity of β-catenin [123].

These data suggest that Osx is an essential component in osteoblast differentiation but has the capability of inhibiting osteoblast proliferation. Thus, Osx guarantees an optimal bone formation rate by strictly regulating osteoblastogenesis [123].

2.3.2.6 Osteoprotegerin

Osteoprotegerin (OPG) is a decoy receptor of RANKL, preventing it to bind to RANK and, thus, inhibiting osteoclasts differentiation [128]. Bone resorption is, therefore, attenuated. OPG is produced by osteoblasts and is essential for regulating osteoclastogenesis. Decreases in the RANKL/OPG ratio results in a decrease of the number of osteoclasts and their activity [128]–[130].

Numerous studies revealed that overexpression of OPG or downregulation of RANKL in mice resulted in reduced osteoclastogenesis, low bone resorption rates and development of osteopetrosis [99], [131]–[133].

2.3.2.7 Semaphorin-3A

Semaphorin-3A (Sema3A) is a membrane-associated secreted protein, from the Semaphorin family, associated with the synchronous regulation of bone resorption and formation [134], [135]. It protects bone by supressing osteoclastic bone resorption and stimulating osteoblastic bone formation [136]. According to Behar *et al.* [137], Sema3A is primarily expressed in osteoblasts, whereas its natural receptor is expressed in osteoclasts.

Several studies demonstrated that Sema3A knockout mice had atypical bone and cartilage development, decreased bone mass or developed osteopenia, as a result of decreased bone formation [138]–[140].

Collectively, these data show that Sema3A plays a critical role in osteoblast differentiation and osteoblastic bone formation, as well as inhibitory functions in osteoclast differentiation and osteoclastic bone resorption.

2.3.3 The Wnt Pathway and Other Bone Turnover Biomarkers

The Wingless/Integrated (Wnt) pathway is a signalling pathway composed by several glycoproteins that are essential for the regulation of bone homeostasis [141]. This pathway is commonly divided into two branches: the canonical pathway, also known as WNT/β-catenin pathway, and the noncanonical pathway.

Two wingless/integrated pathways have been characterized: the canonical Wnt pathway and the noncanonical Wnt pathway. The canonical Wnt/β-catenin pathway, conjointly with BMP signalling, contributes to osteoblast differentiation and skeletal development, leading to bone formation. This canonical path has a negative feedback on osteoclast differentiation and bone degradation, by increasing the expression of OPG [45], [142]. In contrast, the noncanonical pathway stimulates osteoclastogenesis. Wnt signalling is an important regulator of bone remodelling as well, by dictating the fate of MSC differentiation to osteoblasts or to other cell types (Fig. 2.12) [45], [142].

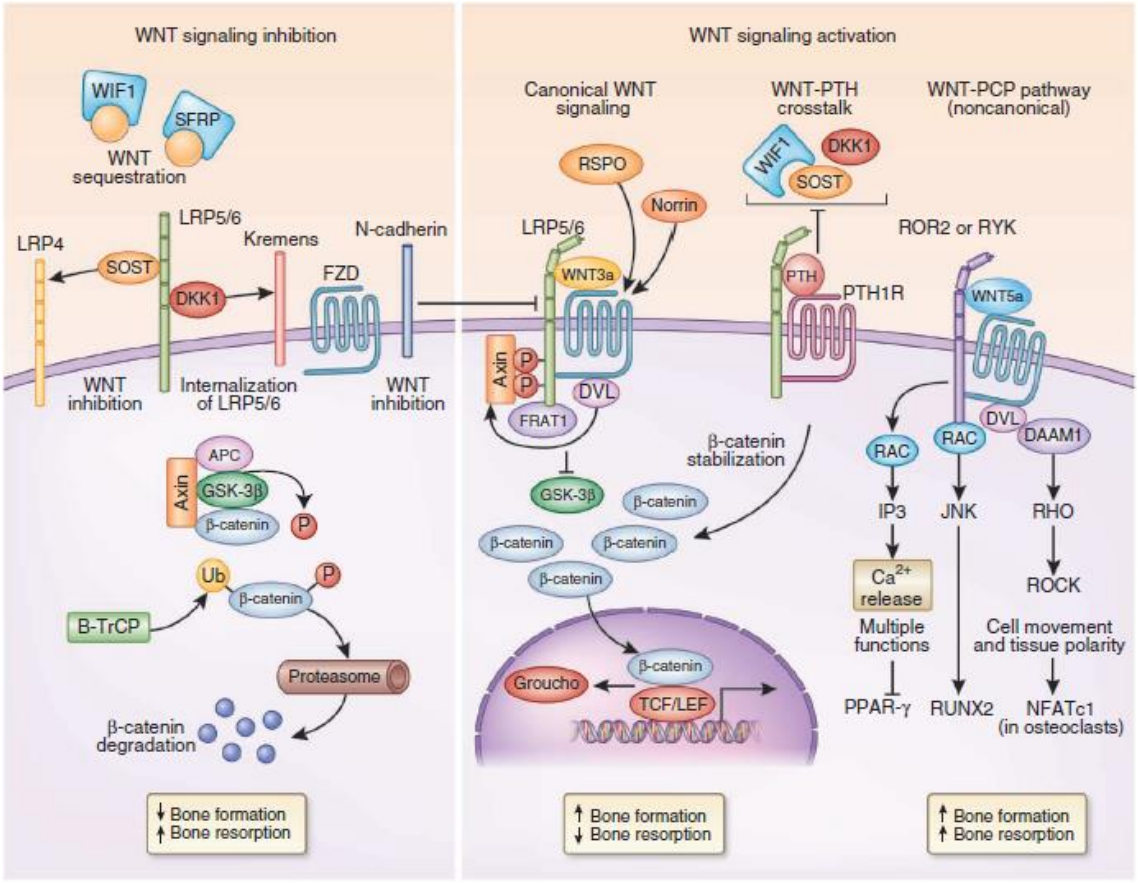


Figure 2.12 – Overview of the two branches of the Wnt pathway. The canonical pathway initiates with the binding process between the canonical Wnt ligands and the Wnt co-receptors LRP5 or LRP6. Wnt signalling is regulated not only by the Wnt ligands but also by its antagonists, namely Dkk1, Dkk2 and SOST. These cytokines indirectly inhibit Wnt signalling by binding to the LRP5/6 proteins, preventing them to stimulate Wnt. Conversely, sFRP and WIF1 are responsible for directly inhibiting both canonical and noncanonical Wnt signalling [142].

Recent studies highlighted the importance of this pathway, crucial for osteoblast differentiation and a master bone mass regulator [143], [144]. In fact, serum levels of dickkopf factor 1 (Dkk1) and sclerostin (SOST), which are negative regulators of Wnt signalling, increase with age and are associated with bone mass loss. Previous studies from the CEDOC group showed that, in fragility fracture patients, osteoblast terminal differentiation is impaired, which is associated with bone mechanical fragility [145]. Moreover, it was also demonstrated that in hip fragility fracture patients there was deterioration of trabecular stiffness, the mechanical parameter directly associated with tissue mineralization [146].

2.3.3.1 Wnt10B

Wnt10b is a specific glycoprotein from the Wnt family that direct and positively correlates with BMD [141]. Studies performed by Bennett and his colleagues [147], [148], showed that elevated expressions of Wnt10b induced bone formation. Consequently, Wnt10b is a good reference indicator for bone formation as it is directly associated with bone mass gain.

2.3.3.2 Dickkopf-1

The dickkopf factor 1 (Dkk1) is a well-known secreted Wnt inhibitor. Its actuation relies on the binding process to the Wnt co-receptors lipoprotein receptor-related proteins 5 and 6 (Lrp5/Lrp6) [141].

Butler *et al.* [144] studied the association between serum levels of Dkk1 and BMD. They found out that expression of Dkk1 was high and negatively correlated with BMD scores and elevated Dkk1 serum levels were traced in osteoporotic patients. Their results, like many others performed in humans and animals [149]–[153], confirm that Dkk1 suppresses osteoblast activity and can be used as a marker for bone metabolism, particularly bone formation.

However, Ueland *et al.* [154] studied bone mass and bone strength associations with cortical and trabecular Dkk1 and SOST levels in Norwegian postmenopausal women and discovered that they were positively correlated with bone mass and strength.

2.3.3.3 Dickkopf-2

Dickkopf factor 2 (Dkk2) also plays a role in the Wnt signalling pathway [141]. The results obtained by Li and his colleagues [155] revealed that mice with null Dkk2 expression had lower bone formation rates, which may suggest a role in bone formation, contrarily to the functions of Dkk1. Furthermore, Rodrigues *et al.* [156] reported that low serum levels of Dkk2 were associated with increased in fracture risk, independently of BMD. Another study demonstrated that when Wnt7b was suppressed, Dkk2 inhibited bone formation, but stimulated late osteoblast differentiation in the presence of Wnt7b [157]. Hence, these data suggest that the effects of Dkk2 on the Wnt pathway strongly depend on the concentration levels of other cytokines.

2.3.3.4 Sclerostin

Sclerostin is a protein encoded by the SOST gene and is mainly produced by osteocytes [141]. Sclerostin binds to Lrp5 and Lrp6, preventing them to stimulate Wnt signalling. Decreased levels of serum sclerostin are associated with increased osteoblast activity, bone formation and bone strength [158], [159], thus, showing that sclerostin is an important negative regulator of bone formation [141]. Moreover, mechanical loading, sensed by osteocytes, is known for inducing bone remodelling episodes and stimulating bone formation [160]. This effect occurs because when mechanical load triggers osteocytes, they suppress the expression of sclerostin, contributing to osteoblast differentiation and proliferation, through Wnt signalling [160].

2.3.3.5 Low-density lipoprotein receptor-related protein 6

Low-density lipoprotein receptor-related proteins (Lrp) are membrane receptors that carry cellular signalling functions in bone [141]. Lrp6 is a co-receptor of Wnt, but has higher affinity for Wnt natural inhibitors, such as sclerostin and Dkk1 [141]. This means that Lrp6 tends to bind with these cytokines to prevent them of inhibiting Wnt. Therefore, Lrp6 plays an ultimate role of stimulating osteoblast differentiation and proliferation, as well as bone formation.

Mani *et al.* [161] showed that mutations in Lrp6 were associated with decreased bone mass and increased fracture risk, as well as other complications, such as coronary

diseases. On the other hand, biochemical evidences were found that demonstrated that Lrp6 helps to improve PTH signalling in osteoblasts, leading to an inhibition of sclerostin, which will, consequently, favour bone formation [75], [141], [162].

2.3.3.6 Secreted frizzled-related protein 1

Secreted frizzled-related proteins (sFRP) are glycoproteins that are related to frizzled proteins, which are receptors that mediate the Wnt signalling pathway [163]. They inhibit Wnt, by competing with membrane-bound frizzled proteins for direct Wnt binding [141], [164] and intervene in bone formation, cartilage development and skeletal disorders [164]. Several researchers reported that mutations in the sFRP1 gene or its deletion, were associated with enhanced trabecular BMD in humans and mice [165], [166].

On the same hand, Yao and his co-workers [167] showed that overexpression of sFRP1 was negatively correlated with BMD and Rodrigues *et al.* [156] showed that high serum levels of sFRP1 were related to an increase in fracture risk. Moreover, Wang *et al.* [164] demonstrated that sFRP1 induced elevated apoptosis rates in osteoblasts, which lead to decreased bone mechanical properties and reduced BMD and trabecular bone volume. These results were similar to those obtained by Bovine *et al.* [168]. Overall, these results clearly suggest that sFPR1 inhibits osteoblasts and bone formation activity, which makes it a well-accepted bone formation marker.

2.3.3.7 Wnt inhibitory factor 1

Wnt inhibitory factor 1 (WIF1) is among the secreted Wnt antagonist that bind directly to Wnt proteins [169]. Its role lies in the ability to change protein conformity to which they bind, thereby preventing their binding to the Wnt receptors [169]. WIF1 is expressed when osteoblasts are in the final phases of their BMP2-induced differentiation and maturation process [141]. Nevertheless, mice with WIF1 null-expression had normal bone development [170], which means that its effect on bone formation inhibition might not be significant on its own.

2.3.3.8 Vitamin D

Vitamin D is produced in the skin and metabolized to 25 hydroxy vitamin D (25(OH) Vitamin D), which is the main circulating form of vitamin D [171], [172]. However, 25(OH) Vitamin D is further metabolized to 1.25$(OH)_2$ Vitamin D, which is the principal hormonal form of vitamin D and responsible for most of its actions [171], [172].

Mice with osteoblastic null-expression of vitamin D receptor (VDR) exhibited elevated levels of ALP and osteocalcin, with increased mineralization rates and bone formation activity [173], [174], while other studies demonstrated that increases in 25(OH) Vitamin D levels were positively correlated with bone formation markers [175]–[177]. Moreover, high expression of VDR in osteoblasts resulted in increased bone mass [178], [179]. Vitamin D deficiency is related to decreased bone mineral density and higher risk of fracture (Fig. 2.13) [180].

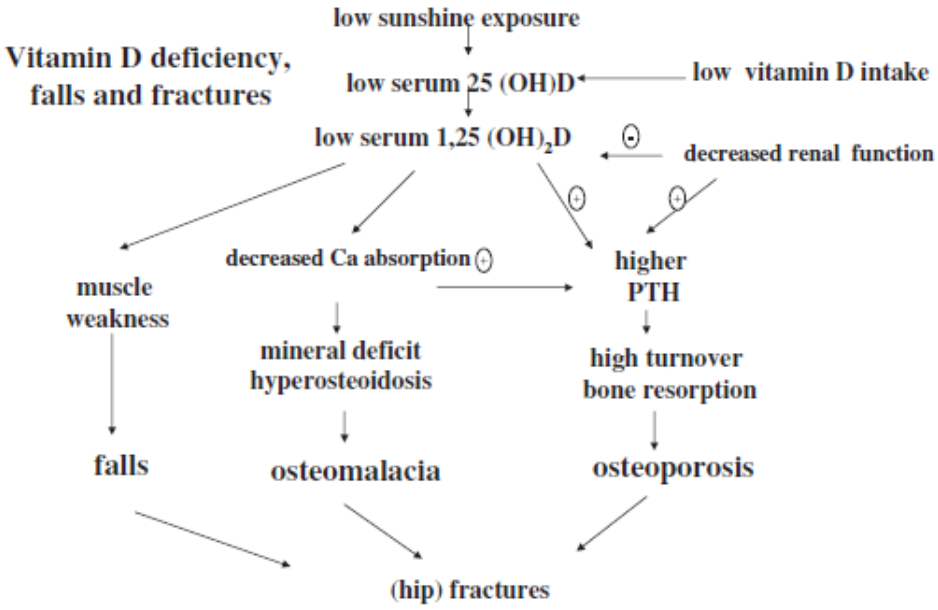


Figure 2.13 – The different pathological pathways of vitamin D deficiency that ultimately lead to fractures [180].

However, 25(OH) Vitamin D can also favour bone resorption activity, by regulating RANKL expression on osteoblasts, thus increasing the number of osteoclasts [171]. Important is to mention that the regulatory effect of 25(OH) Vitamin D in osteoclastogenesis is achieved in osteoblasts [171].

Chapter 3

Methodology

Here, a description of the tools and methods used to meet the objectives is provided. More specifically, it contains information about the study design, data source and study population and for each of the specific objectives, the study variables that were used and the statistical data analysis performed.

3.1 Study Design

The WNT project is a transversal study based on a sample of elderly Portuguese men and women, who underwent surgery due to hip fracture or hip osteoarthritis and aimed to assess which factors were related to the hip fragility fractures. These patients were given total hip replacement surgery between 2008 and 2009, at the orthopedic department of Hospital de Santa Maria in Lisbon. Patient data, which was completely anonymized, was obtained from a clinical database, provided by the Chronic Diseases Research Centre (CEDOC), as part of the WNT project. Since osteoporosis is, essentially, a female related disease, initially, only women were the population-target. However, due to the inexistence of sufficient data, men were also included. Therefore, all 128 patients present in the database were included in the study.

3.2 Model 1: Major Determinants of Bone Mineral Density

3.2.1 Study Variables

As mentioned previously, the most important determinants of bone mineral density found in literature were body weight, body composition and body fat, dietary calcium and vitamin D intake and physical activity. To successfully build a model following these variables to compute missing values and expand the data, matches had to be found from the variables present in the database. Hence, patient weight (in kilogram), patient height (in metres), patient age (in years), sex, body fat (%), current tobacco use (binary: yes or no) and the serum concentration of 25(OH) Vitamin D (in ng/mL) were initially chosen to be fitted into the model.

Tobacco was used as a proxy to physical activity, because Papathanasiou *et al.* [181] reported that smoking was strongly and inversely associated with physical activity in young Greek adults. In addition, Heydari *et al.* [182] also discovered that smoking negatively affected the quality of physical activity in Iranian adults.

Body fat percentage was a result of a mathematical expression proposed by Meeuwsen *et al.* [183], who demonstrated that body fat percentage was strongly correlated with age, sex and body mass index (BMI). These variables were listed in the patient database, which meant that body fat percentage could be calculated. Nevertheless, BMI calculation is based on body weight, which was already considered. Therefore, we chose not to include body fat percentage in our model, but instead utilizing the individual variables (age, sex, height and weight), as recommended by Daniel and Cross [184].

3.2.2 Statistical Analysis

All analyses were carried out using the Statistical Package for the Social Sciences (SPSS, version 24). Linear regression analysis was performed to assess the association between BMD and the major contributors of BMD.

Since linear regression analysis requires normalized residuals [184], Kolmogorov-Smirnov test [184] was computed to ensure that each variable followed a normal distribution. Variables were considered normally distributed if their p-value was higher or equal than 0.05.

For variables which did not follow a normal distribution, mathematical transformations were computed in the following order to normalize the data [185]: logarithmization, exponentiation, cubic root, multiplicative inverse and polynomialization with crescent degrees. Upon analysis, weight and height had to be logarithmized; age suffered a second-degree polynomialization (square age) and vitamin D could not be normalized. As a result, vitamin D was excluded from the model.

During linear regression analysis, the constant was removed from all models, because it was considered that, from a physiological point of view, BMD is inexistent when all variables are equal to zero. Stepwise regression was used with the Backward elimination process. This method includes all initial estimators into the model and tests the deletion of each estimator, according to a model fit criterion [186]. It removes the variable whose loss gives the most statistically insignificant deterioration of the model fit and repeats this process until no further variables can be deleted without a statistically significant loss of fit [186]. The extent to which each variable improved the model, was assessed by its statistical significance (p-value < 0.10).

Bootstrapping, which is a technique for testing model stability, relying on random sampling with replacement [187], was used to externally validate the model. It allows the estimation of the sampling distribution of an estimator, using random resampling with replacement from the original sample [187]. For the purpose of this work, bootstrapping was used to estimate the p-values of the regression coefficients. The age coefficient was multiplied by ten thousand (10 000) to be in the same order of magnitude as the remaining coefficients.

3.3 Model 2: Serum Levels of Bone Turnover Biomarkers

3.3.1 Study Variables

Regarding the serum levels of bone turnover biomarkers, all those available in the database were used. These included osteocalcin (OCL, in ng/mL), bone-specific alkaline phosphatase (BSALP, in U/L), procollagen type 1 N-terminal propeptide (P1NP, in ng/mL), C-terminal telopeptide of type I collagen (CTX-I, in ng/mL), soluble receptor activator of nuclear factor-KB ligand (s-RANKL, in pg/mL), soluble osteoprotegerin (s-OPG, in pg/mL), soluble dickkopf factor 1 (s-Dkk1, in pg/mL), soluble dickkopf factor 2 (s-Dkk2, in ng/mL),

soluble sclerostin (s-SOST, in pg/mL), vitamin D (25(OH) Vit D, in ng/mL) and tartrate-resistant acid phosphatase (TRAP, in U/L). In addition, three clinical diagnostic variables were also included in the model: rheumatoid arthritis (binary: yes or no), corticoid use (binary: yes or no) and secondary osteoporosis (binary: yes or no). From the patient demographic characteristics, sex was included as well.

3.3.2 Statistical Analysis

The same statistical analysis described in 3.2.2 was used to evaluate the association between serum levels of bone turnover biomarkers and BMD. Following the same rationale, BSALP, P1NP, s-SOST and TRAP had to be logarithmized; s-RANKL, s-OPG and s-DKK1 were transformed into their cubic root. OCL, CTX-I, s-Dkk2 and 25(OH) Vitamin D were excluded from the model.

However, because of the existence of certain variables with several missing values, linear regression had to be previously performed to compute the correlations between the different variables, before evaluating their association with BMD. Sex was accounted for in these correlations. After the imputation of the missing values, all variables were given a second Kolmogorov-Smirnov test to ensure that each variable followed a normal distribution. As a consequence, all variables suffered the same transformations as described above, with the exception of TRAP, which had to be excluded from the model.

Summing up, from the initial eleven variables included in the model, six variables were used (BSALP, P1NP, s-SOST, s-RANKL, s-Dkk1 and s-OPG) and the remaining variables were excluded.

3.4 Model 3: Genetic Expression of Bone Turnover Biomarkers

3.4.1 Study Variables

Concerning the genetic expression of bone turnover biomarkers, these were presented as a comparison to a housekeeping (HK) control gene [145]. Apart from one variable (COL1/PMM1), all genetic expression of bone turnover biomarkers available in the database were used as well. These included core-binding factor alpha 1 (Cbfa1/HK), osteoprotegerin (OPG/HK), receptor activator of nuclear factor-KB ligand (RANKL/HK), Osterix

(Osx/HK), alkaline phosphatase (ALP/HK), osteocalcin (OCL/HK), sclerostin (SOST/HK), dickkopf factor 1 (Dkk1/HK), dickkopf factor 2 (Dkk2/HK), Low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/HK and LRP6/HK), secreted frizzled-related protein 1 (sFRP1/HK), Wnt inhibitory factor 1 (WIF1/HK), Wnt10b (WNT10B/HK) and, finally, Semaphorin-3A (SEMA3A/HK). The same three clinical diagnostic variables described in 3.3.1 were included in the model as well. From the patient demographic characteristics, sex was also included.

3.4.2 Statistical Analysis

Since the data presented itself with a wide range of values, often with quite a difference between them, it was considered that, for each variable, all values above two standard deviations from the mean were outliers and, therefore, excluded. In addition, a patient was considered as an outlier patient whenever more than 50% of its variables met the above criteria. As a result, two patients were excluded. Moreover, one variable (sFRP1) had the value zero, which was also excluded from the model.

Once more, because of the existence of certain variables with several missing values, the same statistical analysis described in 3.3.2 was used. Following the same basis, RANKL/HK, OCL/HK, Dkk2/HK, sFRP1/HK and WIF1/HK had to be logarithmized; LRP6/HK and SEMA3A/HK were transformed into their cubic root. Osx/HK, cbfa1/HK, OPG/HK, ALP/HK, SOST/HK, Dkk1/HK, LRP5/HK and WNT10B/HK were excluded from the model.

After the imputation of the missing values and the following Kolmogorov-Smirnov test, all variables suffered the same transformations as described above, apart from WIF1/HK, which was transformed into its cubic root.

Summing up, from the initial fifteen variables included in the model, seven variables were used (RANKL/HK, OCL/HK, Dkk2/HK, LRP6/HK, sFRP1/HK, WIF1/HK, and SEMA3A/HK) and the remaining variables were excluded.

3.5 Graphical User Interface

A graphical user interface (GUI) was developed using MATLAB (Version 9.5.0, The MathWorks Inc. 2018b) in addition to this work. The main purpose to achieve from this GUI is to promote a faster, easier and better way to interact with the user. As an intuitive and user-friendly interface, it is meant to facilitate the visualization and analysis of the results obtained to draw conclusions.

In order to meet some of the proposed requirements, gathered during the project, a GUI was designed, created and later tested to fulfil every given suggestion.
Both equations later achieved in this dissertation (see Chapter 4) were computed into MATLAB, functioning as a calculation engine and linked to the GUI to produce a certain result, which in this case was the value of the bone mineral density. The user can introduce the values of the respective bone biomarkers and the demographic characteristics of the patient, which represent the input values of the system. Afterwards, with a push of a button, the calculation engine runs and produces a result, which can be seen in the GUI. Figure 3.1 shows how these three main blocks communicate with each other and how the information flows during in the system.

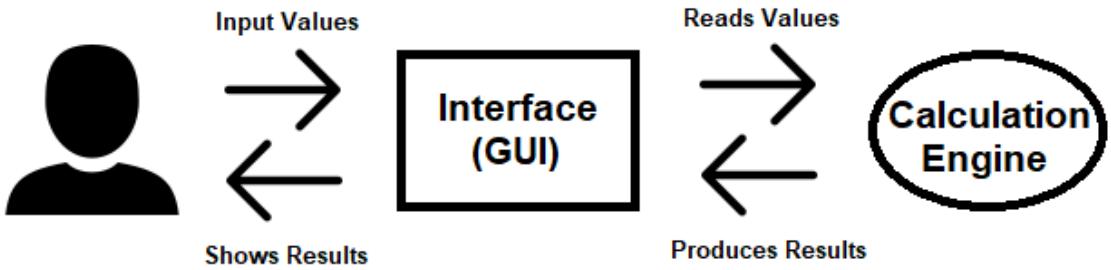


Figure 3.1 – Illustrative schematic of the communication between the main blocks of the system.

Chapter 4

Results

4.1 Patient Characteristics

From the total 128 evaluated patients, 45 were males and 83 were females. In the male group, 11 had surgery as a result of hip fracture and 34 because of hip osteoarthritis, whereas in the female group, 49 had surgery as a consequence of hip fracture and 34 because of hip osteoarthritis (Fig. 4.1). Women were slightly older (76±10) than men (71±10) and had a lower number of smokers (only 6% against 27.3%). Men presented themselves with higher hip BMD (0.84±0.12) compared to women (0.69±0.11). All BMD measurements (Hip BMD) were a result from DEXA. Figures 4.2 to 4.5 show the demographic characteristics from both groups regarding age, weight, height and hip BMD.

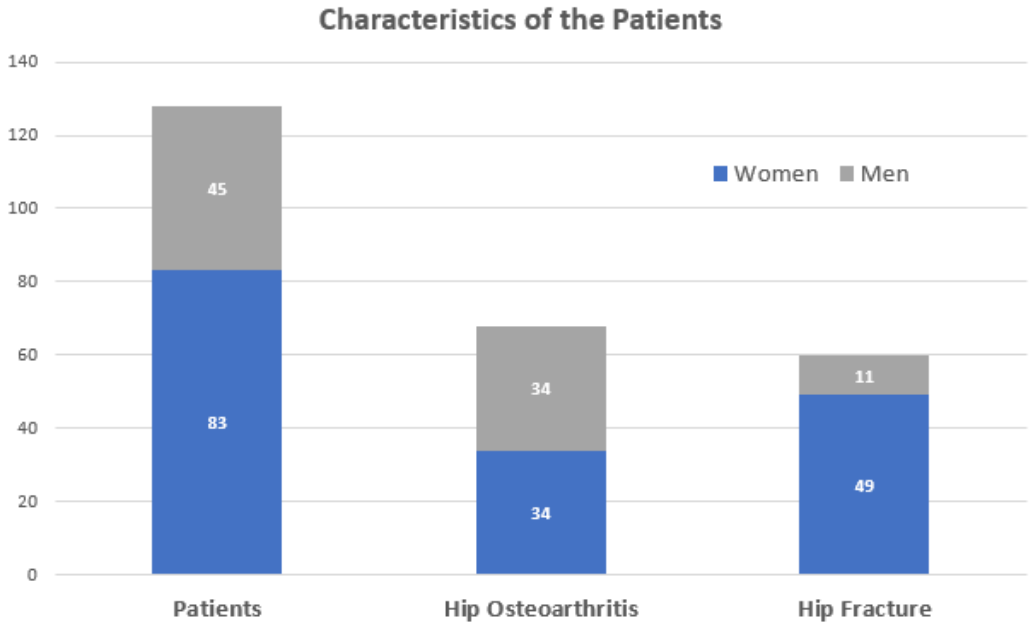


Figure 4.1 – Total number of male and female patients, as well as the number of discriminated patients with respect to the type of surgery given.

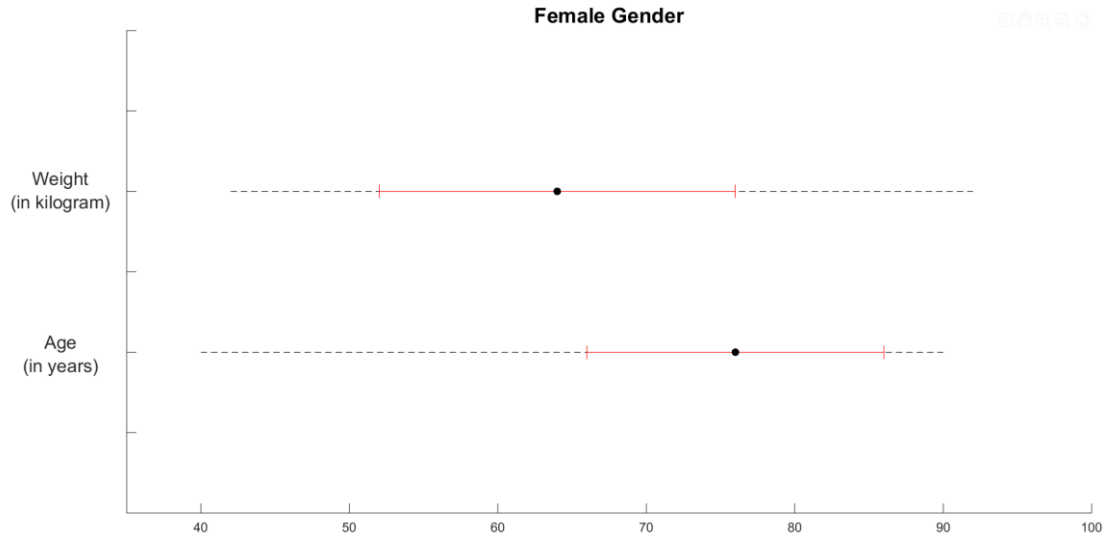


Figure 4.2 – Demographic characteristics regarding age and weight in the female group. Mean is represented by the single black dots; the experimental standard deviation (SD) is represented by the red bars; minimum and maximum values are represented by the black dashed lines. With respect to age, values ranged between 40 and 90 years old (yo) with 76±10 yo (Mean±SD). With respect to weight, values ranged between 42 and 92 kilogram (kg) with 64±12 kg (Mean±SD).

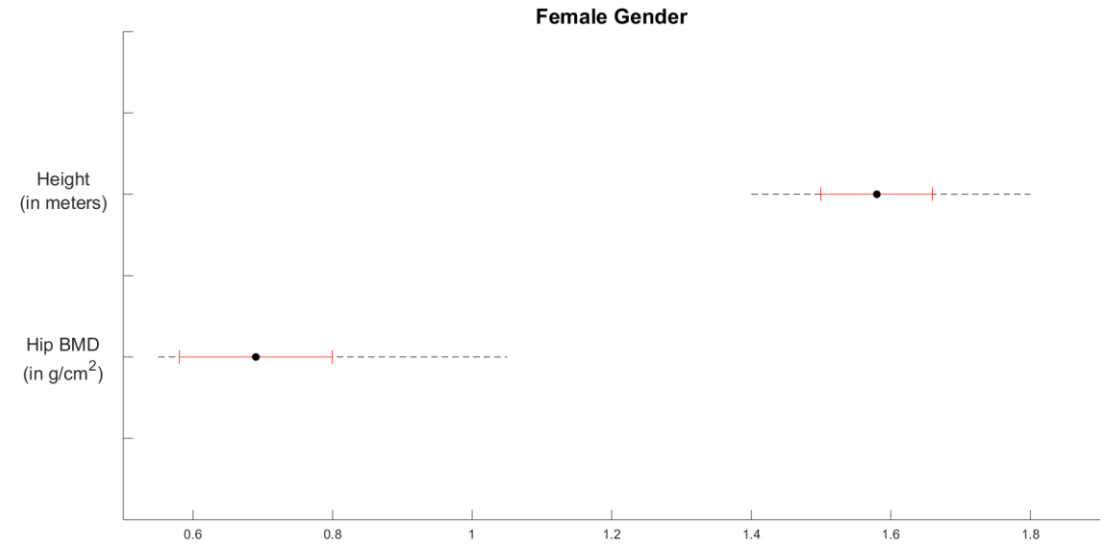


Figure 4.3 – Demographic characteristics regarding height and hip BMD in the female group. Mean is represented by the single black dots; the experimental standard deviation (SD) is represented by the red bars; minimum and maximum values are represented by the black dashed lines. With respect to height, values ranged between 1.4 and 1.8 meters (m) with 1.58±0.08 m (Mean±SD). With respect to hip BMD, values ranged between 0.55 and 1.05 g/cm^2 with 0.69±0.11 g/cm^2 (Mean±SD).

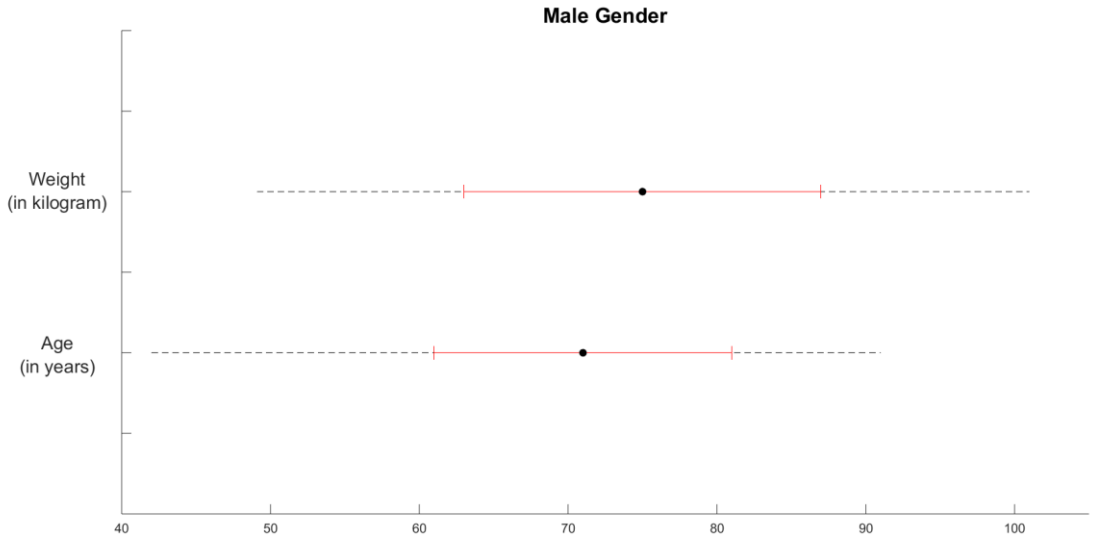


Figure 4.4 – Demographic characteristics regarding age and weight in the male group. Mean is represented by the single black dots; the experimental standard deviation (SD) is represented by the red bars; minimum and maximum values are represented by the black dashed lines. With respect to age, values ranged between 42 and 91 years old (yo) with 71±10 yo (Mean±SD). With respect to weight, values ranged between 49.2 and 101 kilogram (kg) with 75±12 kg (Mean±SD).

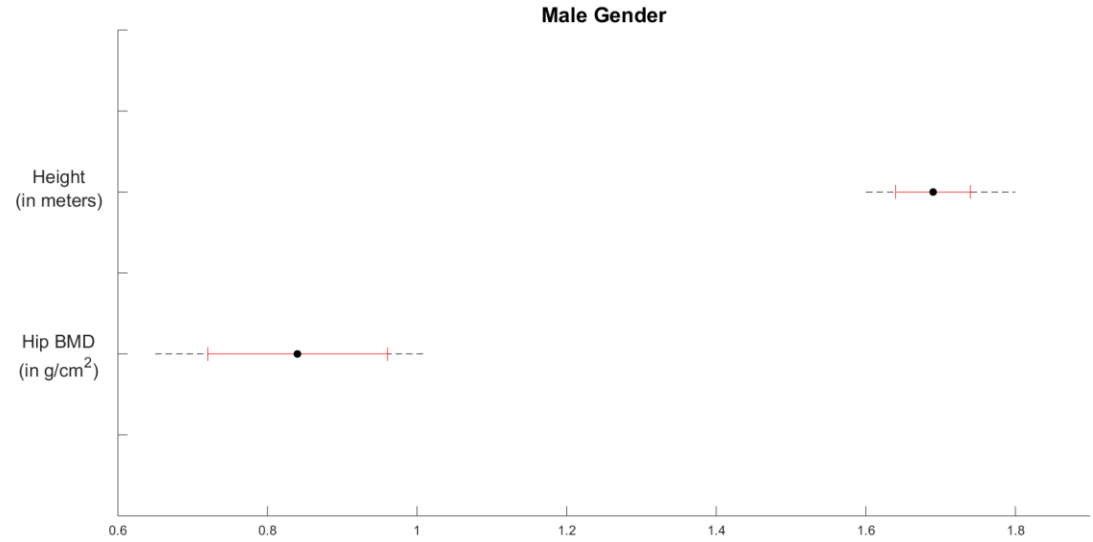


Figure 4.5 – Demographic characteristics regarding height and hip BMD in the female group. Mean is represented by the single black dots; the experimental standard deviation (SD) is represented by the red bars; minimum and maximum values are represented by the black dashed lines. With respect to height, values ranged between 1.6 and 1.8 meters (m) with 1.69±0.05 m (Mean±SD). With respect to hip BMD, values ranged between 0.65 and 1.01 g/cm^2 with 0.84±0.12 g/cm^2 (Mean±SD).

The prevalence of rheumatoid arthritis and secondary osteoporosis was higher in the female group, with 8.4% in both cases. Moreover, seven women (8.4%) were on corticoid therapy, in contrast with men, which only had one patient on corticoid therapy.

Concerning the serum concentrations of bone turnover biomarkers, women had, in general, higher values compared to men (Table 4.1). Osteocalcin was the only factor that was more elevated in the men group (9.5±10.6) than in the female group (8.6±8.5).

On the other hand, the RANKL/OPG ratio was nearly the same in both groups, with a value of approximately 0.123. The difference between maximum and minimum values were greater in P1NP, CTX-I, s-OPG, s-Dkk1 and s-SOST, with CTX-I having the highest dispersion of values (0.1 - 898.1 in the men group and 0.1 - 1628.4 in the female group).

Table 4.1 – Characterization of the serum levels of bone turnover biomarkers in the male and female groups (with missing values). Data shown as Mean ± Experimental standard deviation.

	Males			Females		
	Mean ± SD	Min.	Max.	Mean ± SD	Min.	Max.
Serum Levels						
OCL (ng/mL)	9.5 ±10.6	2.0	45.4	8.6 ± 8.5	2.0	30.8
ALP (μg/L)	11.5 ± 6.8	3.9	30.3	12.2 ± 4.7	5.0	20.2
P1NP (ng/mL)	58.1 ± 65.0	10.1	310.0	77.0 ± 110.8	10.6	854.5
CTX-I (ng/mL)	50.5 ± 160.6	0.1	898.1	72.1 ± 234.4	0.1	1628.4
s-RANKL (pg/mL)	12.3 ± 8.9	1.0	26.8	14.9 ± 13.2	0.4	54.3
s-OPG (pg/mL)	100.1 ± 75.1	2.4	311.2	120.9 ± 89.4	0.9	412.6
s-Dkk1 (pg/mL)	472.1 ± 209.1	25.7	1025.5	663.8 ± 343.5	103.2	1762.2
s-Dkk2 (ng/mL)	6.5 ± 9.8	0.1	51.6	11.4 ± 16.3	0.1	75.0
s-SOST (pg/mL)	380.4 ± 198.3	109.8	869.6	383.9 ± 201.2	63.7	985.3
25(OH) Vit D (ng/mL)	27.9 ± 22.9	11.4	85.8	28.3 ± 26.3	11.89	142.2
TRAP (U/L)	2.2 ± 1.7	0.4	6.2	2.4 ± 1.9	0.5	10.0

With respect to the genetic expression of bone turnover biomarkers, the same pattern found in table 4.1 can be observed in table 4.2. Women had higher bone biomarker expression than men, apart from OCL, WIF1 and WNT10B. Expressions of LRP5 and LRP6

were found to be approximately the same in both groups (0.2±0.3 and 0.2±0.4 in men and women for LRP5, respectively, and 0.3±0.3 and 0.3±0.5 in men and women for LRP6, respectively).

Widest ranges were found in OPG, RANKL, Osx, SOST, Dkk1 and WNT10B, with SOST having the highest dispersion of values (0.006 - 522.6 in men and 0.0005 - 660.8 in women).

Table 4.2 – Characterization of the bone turnover biomarkers in the male and female groups (with missing values). Data shown as Mean ± Experimental standard deviation.

	Males			Females		
	Mean ± SD	Min.	Max.	Mean ± SD	Min.	Max.
Gene Expression						
Cbfa1/HK	0.2 ± 0.2	0.005	1.3	0.3 ± 0.8	0.001	5.8
OPG/HK	1.5 ± 6.4	0.004	39.9	5.8 ± 23.9	0.002	143.3
RANKL/HK	3.8 ± 13.7	0.002	77.0	9.0 ± 28.2	0.005	193.1
Osx/HK	4.4 ± 16.7	0.003	102.7	11.7 ± 41.4	0.002	270.6
ALP/HK	0.2 ± 0.3	0.009	1.2	0.3 ± 0.6	0.001	4.4
OCL/HK	0.1 ± 0.5	0.00004	2.6	0.1 ± 0.2	0.00002	1.1
SOST/HK	17.0 ± 82.7	0.006	522.6	34.1 ± 109.0	0.0005	660.8
Dkk1/HK	1.2 ± 6.8	0.00002	39.7	9.4 ± 40.5	0.00007	225.0
Dkk2/HK	0.4 ± 0.9	0.0000007	4.1	1.6 ± 5.6	0.002	32.3
LRP5/HK	0.2 ± 0.3	0.0002	1.6	0.2 ± 0.4	0.002	2.1
LRP6/HK	0.3 ± 0.3	0.00005	1.4	0.3 ± 0.5	0.00006	2.7
sFRP1/HK	1.6 ± 5.6	0.0037	34.4	2.2 ± 5.4	0.0006	29.7
WIF1/HK	11.7 ± 17.6	0.033	62.7	9.1 ± 15.5	0.003	64.3
WNT10B/HK	38.0 ± 89.0	0.000005	399.2	32.3 ± 71.7	0.0003	334.4
SEMA3A/HK	0.3 ± 0.3	0.0008	1.1	0.4 ± 0.5	0.009	2.1

4.2 Model 1: Major Determinants of Bone Mineral Density

Estimating the association between BMD and the major determinants of bone mineral density was necessary to compute nighty-seven missing values in the database. The model achieved (Table 4.3) had an associated R-square of 0.984, was validated with the bootstrapping technique (thousand iterations) and had a mean percent error of 6.8%. Concerning the gender estimator, females were categorized as value one and males were categorized as value zero.

Table 4.3 – Linear regression analysis of major determinants of BMD associated with bone mineral density.

Model 1	Coefficient	Without Bootstrap p-value	With Bootstrap p-value
Sex	-0.101	0.024	0.111
Age^2	-0.2238	0.089	0.328
$\ln Weight$	0.222	<0.001	0.001
R-square		0.984	

4.3 Model 2: Serum Levels of Bone Turnover Biomarkers

Model 1 allowed the computation of all BMD missing values, which was extremely important for achieving more robust models that would follow. The same principle was applied to all variables that were considered in the models. Table 4.4 demonstrates the equations that were reached through linear regression analysis. These equations were used to compute missing values among the serum levels of bone turnover biomarkers.

After computing the missing values for the serum levels of bone turnover biomarkers, it was possible to estimate the association between BMD and the concentrations of these regulatory factors. This estimation is represented by model 2.1, which can be seen in table 4.5.

Model 2.1 was validated with the bootstrapping technique, with nine hundred and seventy-three iterations. However, upon a careful analysis of the obtained p-values, it was

observed that two of the seven variables were not statistically significant with bootstrapping validation (table 4.5). Consequently, these two variables (rheumatoid arthritis and corticoid use) were removed from the model, which allowed the achievement of the final model (model 2.2), with a R-square of 0.986 and validated with bootstrapping as well (table 4.6).

Table 4.4 – Linear regression analysis for serum bone biomarkers to compute missing values.

Model	Coefficient	p-value	R-square
ALP			0.910
$\ln P1NP$	5.441	<0.001	
$\ln SOST$	-1.394	0.067	
P1NP			0.839
Sex	27.027	0.055	
$\sqrt[3]{Dkk1}$	-8.692	0.007	
$\ln ALP$	44.361	<0.001	
RANKL			0.839
$\ln TRAP$	-5.831	0.049	
$\ln ALP$	9.569	<0.001	
OPG			0.813
$\sqrt[3]{Dkk1}$	15.523	0.007	
$\ln ALP$	37.351	0.077	
$\sqrt[3]{RANKL}$	-43.412	0.047	
Dkk1			0.796
$\sqrt[3]{OPG}$	109.486	<0.001	
SOST			0.872
$\ln TRAP$	-134.196	0.009	
$\ln P1NP$	120.481	<0.001	
TRAP			0.734
$\ln ALP$	1.027	<0.001	

Table 4.5 – Linear regression analysis of serum bone biomarkers associated with bone mineral density.

Model 2.1	Coefficient	Without Bootstrap p-value	With Bootstrap p-value
Sex	-0.180	<0.001	0.001
Rheumatoid arthritis	0.108	0.072	0.126
Secondary Osteoporosis	-0.164	0.003	0.017
Corticoid use	0.089	0.099	0.113
$\ln \mathrm{P1NP}$	0.028	0.018	0.015
$\ln SOST$	0.103	<0.001	0.001
$\sqrt[3]{Dkk1}$	0.018	0.007	0.008
R-square		0.987	

Table 4.6 – Linear regression analysis of serum bone biomarkers associated with bone mineral density, after bootstrap validation.

Model 2.2	Coefficient	p-value
Sex	-0.174	<0.001
Secondary Osteoporosis	-0.057	0.108
$\ln \mathrm{P1NP}$	0.019	0.090
$\ln SOST$	0.102	<0.001
$\sqrt[3]{Dkk1}$	0.023	<0.001
R-square		0.986

4.4 Model 3: Genetic Expression of Bone Turnover Biomarkers

Following the same basis as in the serum bone biomarkers, linear regression analysis was performed to compute missing values in the genetic expression of bone biomarkers (table 4.7). Afterwards, model 3 was built to find out whether gene expression of bone biomarkers could successfully estimate an association with bone mineral density. The model was validated with the bootstrapping technique, with thousand iterations. As a result, none of the variables had to be removed. In addition, model 3 had a R-square of 0.967. The results are shown in table 4.8.

Table 4.7 – Linear regression analysis for genetic expression of bone biomarkers to compute missing values.

Model	Coefficient	p-value	R-square
RANKL			0.514
Sex	10.386	0.014	
$\ln OCL$	-3.339	0.001	
$\ln Dkk2$	-1.573	0.058	
$\ln sFRP1$	5.804	<0.001	
OCL			0.646
$\ln Dkk2$	-0.099	<0.001	
$\ln WIF1$	0.050	0.056	
$\sqrt[3]{LRP6}$	0.833	0.002	
$\sqrt[3]{SEMA3A}$	-0.690	0.001	
$\ln RANKL$	0.055	0.003	
Dkk2			0.358
$\ln sFRP1$	0.734	0.002	
$\sqrt[3]{LRP6}$	7.548	<0.001	
LRP6			0.584
$\sqrt[3]{SEMA3A}$	0.549	<0.001	
$\ln OCL$	0.030	0.034	
$\ln Dkk2$	0.029	0.014	
sFRP1			0.524
$\sqrt[3]{SEMA3A}$	1.706	0.043	
$\ln RANKL$	0.800	<0.001	
$\ln OCL$	-0.331	0.035	
WIF1			0.404
$\sqrt[3]{LRP6}$	18.047	<0.001	
SEMA3A			0.686
$\ln sFRP1$	0.04	0.011	
$\sqrt[3]{LRP6}$	0.937	<0.001	

Table 4.8 – Linear regression analysis of genetic expression of bone biomarkers associated with bone mineral density.

Model 3	Coefficient	Without Bootstrap p-value	With Bootstrap p-value
Sex	-0.070	0.044	0.044
Rheumatoid arthritis	-0.191	0.012	0.012
Corticoid use	0.129	0.077	0.077
$\ln OCL$	-0.059	<0.001	<0.001
$\ln Dkk2$	-0.022	0.004	0.004
$\ln sFRP1$	-0.047	<0.001	<0.001
$\sqrt[3]{LRP6}$	0.232	0.028	0.028
$\sqrt[3]{WIF1}$	0.070	<0.001	<0.001
$\sqrt[3]{SEMA3A}$	0.322	0.002	0.002
R-square		0.967	

4.5 Graphical User Interface

Figure 4.2 shows the displacement of the numerous elements throughout the GUI. In addition to what is shown below, a patient database was also created and developed within the GUI, which allows the user to register and record the identification number of the patient, all its measured values of serum levels and genetic expression of bone turnover biomarkers and the number of consults taken.

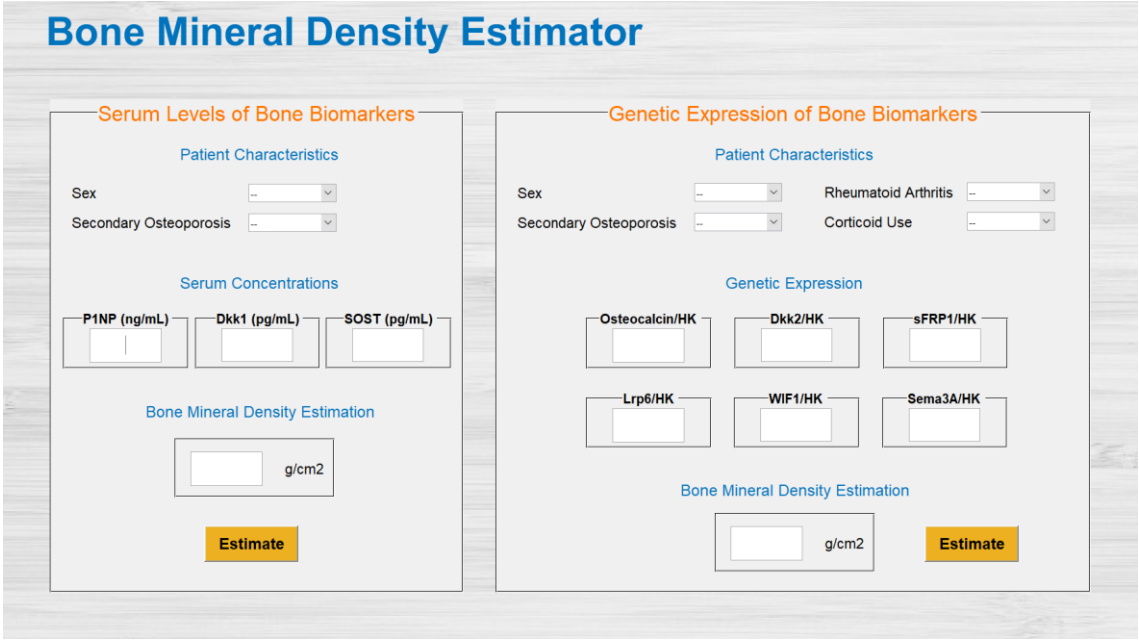


Figure 4.6 – GUI representing a bone mineral density estimator for elderly Portuguese men and women. At the left, the panel that controls the serum levels of bone biomarkers provides the possibility to input the demographic characteristics of the patient and to compute the concentrations of the bone turnover biomarkers. At the right, the panel that controls the genetic expression of bone biomarkers provides the possibility to input the demographic characteristics of the patient as well and to compute the genetic expressions of the bone turnover biomarkers. All the results of bone mineral density estimates are given in g/cm^2.

Chapter 5

Discussion

The results presented mention three different models, that represent a way of estimating the association between bone mineral density and three major groups of estimators, which include the principal determinants of BMD (model 1), serum concentrations of bone turnover biomarkers (model 2.2) and the genetic expression of bone turnover biomarkers (model 3), respectively. All the models were achieved with a stepwise regression method, namely the backward elimination technique, and further validated with bootstrapping. Correlations between estimators within models 2 (2.1 and 2.2) and 3 were performed as a vehicle to diminish the quantity of missing values in those estimators.

5.1 Model 1: Major Determinants of Bone Mineral Density

Model 1 provided a bridge for the achievement of more robust results in models 2 and 3, by allowing the substitution of the missing values by an acceptable BMD estimation. Although the model did not include all initial estimators, it demonstrated a mean percent error of 6.8%.

There was a positive correlation between weight and BMD, which corroborates the findings of Salamat *et al.* [188], who demonstrated that BMD was higher in obese and overweight men, premenopausal and postmenopausal women, compared to those who had normal weight. Silva *et al.* [189] also observed that increased body mass indexes, due to increases in body weight, contributed to a positive influence on BMD in postmenopausal women.

Both overweight and obesity may offer a so-called protective role against bone loss and can be mediated by fat mass and lean mass [189], [190]. Fat mass, similar to muscle mass (lean mass), can induce higher skeletal-loading events, promoting bone formation and,

thus, preventing excessive bone loss [191]. Although it might seem that the protective role of overweight and obesity is mainly induced through this mechanism, there are alternative roots to which fat mass can interfere with bone. It is believed that adipose tissue can contribute to this protective role through the production of oestrogens and other cytokines, such as leptin, that influence osteoblasts and promote bone formation as well [56], [192].

Negative correlations were found between both age and sex and BMD. Age is a vital factor in bone mass loss, mainly in women. Nuti *et al.* [193] reported that postmenopausal BMD changes are closely linked to the years since the onset of menopause, similar to the results obtained by Heidari *et al.* [194]. These results showed that older postmenopausal women had decreased values of BMD compared to early postmenopausal women. Concerning gender, Nieves *et al.* [195] showed that men had greater hip BMD compared to women, despite comparable body sizes. BMD differences between genders can be related with a genetic tendency for males to have higher lean mass percentages compared to females and attaining higher peak bone mass [196].

The model obtained in this work takes into consideration weight, age and sex. After bootstrapping validation, it was observed that sex and age were no longer statistically significant. However, it was opted that those two estimators would remain in the model, since they represent two important determinants of bone mineral density, thus achieving more acceptable results. In addition, this model only represented a way of filling the missing values of BMD in the database and was not the main objective of this dissertation. Therefore, this decision was thought to be appropriate for this context.

The exclusion of the regression constant from the model, which is explained in chapter 3, forced the line that best fitted the data to pass through the origin of the axis. This resulted in a higher value for the associated R-square, which was 0.984. Including the regression constant would significantly decrease the R-square, which would be about 30% to 40% lower. Nevertheless, this model can be indirectly comparable (because it has no constant) to a few models obtain by several authors. Baheiraei et al. [197] obtained an R-square of 0.38 for femoral neck BMD in Iranian women, using a model with age, BMI and tobacco use as dependent variables. Rexhepi et al. [198] reported R-squares of 0.371 and 0.372 for BMD associations with age-adjusted weight and BMI, respectively, in Russian menopausal women. On the other hand, Salamat et al. [199] could only explain 14% of changes in BMD accounting for age-adjusted weight and BMI. Talash et al. [200] had an R-square of 0.308 for the changes in femoral neck BMD, using BMI as estimator.

Meybodi et al. [201] achieved R-squares of 0.21 and 0.25 for femoral neck BMD and total femur BMD, respectively, considering age, BMI, current smoking and current physical activity.

5.2 Model 2: Serum Levels of Bone Turnover Biomarkers

In order to achieve more robust results in models 2.1 and 2.2, by substituting as much as missing values as possible, linear regression analysis was first performed to produce equations that represented the correlations between the different estimators. The computation of missing values for each variable was a necessary step, but far from representing the real measured values of the patients. This imputation of missing values may have contributed to the appearance of wrong associations between variables, leading to contrary results of what was expected.

Model 2.2 provided an estimation of bone mineral density, based on the serum concentrations of bone turnover biomarkers. Although the final model only accounted for three bone biomarkers, it still produced promising results, with an R-square of 0.986.

Originally, model 2.1 comprised seven estimators, from which four were demographic characteristics of the patients and the remaining three were serum concentrations of bone biomarkers. Nonetheless, after applying bootstrapping, two estimators, namely rheumatoid arthritis and corticoid use, were no longer statistically significant, leading to their exclusion from the model. Thus, the final model (model 2.2) was composed by sex, secondary osteoporosis, P1NP, SOST and Dkk1.

Negative correlations were found between both sex and secondary osteoporosis and BMD. A negative association between sex and BMD is in concordance with the findings of Nieves *et al.* [195] and Alswat *et al.* [196], as mentioned previously. Regarding secondary osteoporosis, it is "defined as low bone mass with microarchitectural alterations in bone leading to fragility fractures in the presence of an underlying disease or medication", according to Mirza *et al.* [202]. Secondary osteoporosis has been considered a clinical risk factor for osteoporosis [10], which means that whether the patient has or not secondary osteoporosis can be linked to higher or lower BMD values. More specifically, a patient with secondary osteoporosis is most likely to have decreased values of BMD, which agrees with the results obtained in the model.

There was a positive association between P1NP and BMD, which corroborates the findings of Szulc *et al.* [120]. P1NP is considered a bone formation biomarker and its serum

concentrations are correlated with elevated bone formation rates [120]. However, these results contrast with those reported by Elma *et al.* [122], who discovered elevated serum levels of P1NP in osteoporotic women. This apparent contradictory particularity might be caused by the inhibition of the activity of mature osteoblasts by other cytokines responsible for osteoblast regulation. P1NP derives from the cleavage process of the collagen type I molecule, by the activity of specific proteases at the N-terminal [94]. This process has its origins mainly from proliferating osteoblasts, which are not fully mature, yet [94]. Therefore, P1NP continues to be produced but the inhibition of the mature osteoblasts prevents the deposition of bone matrix. Together with increased osteoclast activity, these factors may explain why Elma and her co-workers [122] discovered elevated serum concentrations of P1NP in osteoporotic women.

Positive correlations were also found in both SOST and Dkk1 and BMD. Sclerostin and Dkk1 are two well-known WNT antagonists, that bind to LRP5 and LRP6 proteins, preventing them to stimulate the WNT signalling pathway. Elevated serum levels of SOST and Dkk1 have been linked to osteoporotic patients [144], [203], [204]. However, several authors reported positive correlations between serum sclerostin levels and BMD in postmenopausal women. For instance, Polyzos *et al.* [205] observed that serum sclerostin was decreased in women with postmenopausal osteoporosis compared with non-osteoporotic postmenopausal women. Xu and his colleagues [206] also obtained the same results as Polyzos *et al.* and demonstrated that serum sclerostin levels were positively correlated with BMD. Moreover, Reppe *et al.* [207] showed that both serum sclerostin levels and bone SOST mRNA expression were positively correlated with total hip BMD. Ueland *et al.* [154] reported positive correlations between both Dkk1 and sclerostin and bone mass and bone strength in postmenopausal osteoporotic women.

Underlying pathologies may also be part of this positive association, since Cejka *et al.* [208] discovered a positive correlation between serum sclerostin levels and bone mineral density in haemodialysis patients. Although sclerostin has an inhibitory role in bone formation, it might be affected by other cytokines, as part of compensatory counteracting mechanisms, lowering serum sclerostin concentrations and showing the observed positive relations with BMD [207]. Despite some disagreement between results and biological evidences, the results obtained by the model can be accepted for sclerostin, but not for Dkk1. Possible explanations may lie in the data itself, since the mathematical transformations do not interfere with the sign of the correlation (logarithm and cubic root are crescent/increasing functions). It was observed that most of the variables present in the database had values with extremely wide ranges (see chapter 4,

table 4.2), indicating eventual human input errors, such as measurement units, or underlying pathologies not mentioned in the database.

Overall, the results obtained in model 2 are, to a certain part of extent, promising but embrace a low number of bone biomarkers and need a careful examination on the Wnt regulators, namely the positive correlation found between Dkk1 and BMD.

5.3 Model 3: Genetic Expression of Bone Turnover Biomarkers

Following the same rationale as in models 2.1 and 2.2, linear regression analysis was first performed to produce equations that represented the correlations between the different estimators, in order to substitute as many missing values of the genetic expression of bone turnover biomarkers as possible. As it happened with the serum concentrations of bone biomarkers, the computation of missing values for each genetic expression of bone biomarker was a necessary step, but far from representing the real measured values of the patients. This imputation of missing values may have contributed to the appearance of wrong associations between variables, leading to contrary results of what was expected.

The last model (model 3) was able to grant a valuable and considerable promising estimation of bone mineral density, based on the genetic expression of bone turnover biomarkers (R-square of 0.967). Only two estimators were excluded from the model, namely secondary osteoporosis and RANKL/HK, which indicated a good overall fitting of the data.

Corticoid use, Lrp6, WIF1 and Sema3A were positively correlated with BMD. The positive relationship between corticoid use and BMD was not expected, since it is documented that the use of corticosteroids has a significant negative impact on BMD [209]–[211]. Walsh *et al.* [212] also discovered that either oral or inhaled corticosteroids had a negative correlation with BMD. This positive correlation found in the model may have derived from the substitution of the missing values of BMD. Model 1 did not have into consideration the effect of corticoid therapy, because it was not a major determinant of BMD. However, the introduction of the estimated BMD values into the database may have created a wrong correlation between BMD and corticoid use.

The positive correlation between Sema3A and BMD agrees with the studies performed in mice, demonstrating that Sema3A knockout-mice had decreased bone formation and bone mass [137]–[139]. However, Liu *et al.* [213] did not find any significant differences

between Sema3A expression and BMD in Chinese postmenopausal women. These results indicate that, even if mice trials have been successful in identifying correlations between bone mineral density and Sema3A, human trials have not yet been able to assess this association, leading to a certain critical sense towards the correlation found in the model.

WIF1 was positively correlated with BMD. This result was not expected, since WIF1 is linked to the inhibition of the Wnt pathway, resulting in low bone mass. To illustrate this, Yavropoulou *et al.* [214] demonstrated that the serum microRNA expressing the WIF1 gene was 14.76-fold higher in women with low bone mass compared to controls. However, studies in WIF1 knockout-mice showed normal bone development [170]. Nevertheless, the positive association found between WIF1 and BMD may be related, once again, to the substitution of the missing values of BMD. The introduction of the remaining estimated WIF1 values into the database may have created a wrong correlation between BMD and the estimator.

Lrp6 was also positively correlated with BMD, confirming the results obtained by Mani *et al.* [161], who demonstrated that mutation in the Lrp6 gene lead to low bone mass and increased fracture risk. LRP6 protein bind to the natural Wnt inhibitors, allowing the stimulation of the pathway and consequent osteoblast differentiation and proliferation, as well as bone formation.

In contrast, negative correlations were found between sex, rheumatoid arthritis, osteocalcin, Dkk2 and sFRP1 and BMD. Sex has already been mentioned to favour men over women concerning BMD [195], [196]. Regarding rheumatoid arthritis, the obtained relationship is in concordance with the findings of Heidari *et al.* [215], Hafez *et al.* [216] and Lodder *et al.* [217]. These authors reported significant negative correlations between the damages caused by the disease and low BMD.

Dkk2 was found to have a negative association with BMD, which agrees with the results obtained by Li *el al.* [155], who revealed that mice with null Dkk2 expression had lower bone formation rates. Li *et al.* [157] also demonstrated that Dkk2 stimulated late osteoblast differentiation in the presence of Wnt7b.

A negative correlation was also found between sFRP1 and BMD, which can be validated with the results obtained by several researchers [164]–[168]. Some authors reported that upregulating sFRP1 expression induced osteoblast apoptosis and the inhibition of osteoblast activity, resulting in decreased bone properties and BMD, while others showed that the deletion of the sFRP1 gene was associated with increased BMD.

Finally, the negative correlation of osteocalcin with BMD obtained in the model is confirmed by the results of Singh *et al.* [113], who reported negative correlations between osteocalcin levels and BMD in postmenopausal women. In addition, Ducy *et al.* [112] verified that mice with null expression of osteocalcin had higher bone mass and bone formation rates.

Overall, the results obtained in model 3 indicate that an upregulation of the Wnt pathway regulators, namely some of the Wnt inhibitors, is associated with low bone mass, which reinforces the findings of Rodrigues *et al.* [145], [156].

Despite the achievement of promising results, the models do present some limitations. To begin with, the amount of data in which the models are based on is quite small and does not represent a strong basis to withdraw conclusions. Another drawback is related to the choice of the removal of the constant from all models. This removal was justified, from a physiological point of view, as bone mineral density was considered to be inexistence if all estimators were equal to zero. However, in the scientific community, the removal of this constant is seldomly seen. The constant modulates the error associated to the model itself, but in this case the error is intrinsic to the variables, meaning that all the model coefficients have some extent of this associated error.

Chapter 6

Conclusions

6.1 Final Remarks

The main objective of this work was to assess the association between bone gene expression and serum concentrations of markers of osteoblast and osteoclast differentiation and of Wnt pathway regulators with bone mineral density. Developing a method capable of estimating bone mineral density based on its association with bone biomarkers may help to reduce both the need of *in vivo/in vitro* studies and the frequency of DEXA exams prescribed to diagnose osteoporosis. Two types of estimators were used, namely the serum levels of bone biomarkers and their genetic expression.

As a first approach, the association between bone mineral density and its major determinants was studied, in order to compute missing values in the patient database. Matches were found between the major determinants of bone mineral density found in the literature and the available variables in the database. Sex was included in the model as well. Since linear regression was used to estimate this association, all variables were taken a Kolmogorov-Smirnov test to ensure that each variable followed a normal distribution. For those which did not follow a normal distribution, mathematical transformations were computed in the following order to normalize the data: logarithmization, exponentiation, cubic root, multiplicative inverse and polynomialization with crescent degrees. During linear regression analysis, the constant was removed from all models and stepwise regression was used with the Backward elimination process. Bootstrapping was used to externally validate the model.

Upon the computation of the bone mineral density missing values, the relationship between the variables representing the serum levels of bone turnover biomarkers was assessed to compute missing values within these variables. The same rationale concerning the statistical analysis was applied. Afterwards, the association of serum levels of bone

biomarkers with bone mineral density was estimated. Besides all bone biomarkers, four variables were included to the model, namely sex, rheumatoid arthritis, corticoid use and secondary osteoporosis. The final model, which was validated with bootstrapping, accounted for two demographic variables (sex and secondary osteoporosis) and three variables of serum levels of bone biomarkers (P1NP, SOST and Dkk1) and achieved an R-square equal to 0.986.

With respect to the genetic expression of bone turnover biomarkers, the same procedure as in the serum levels of bone biomarkers was conducted. Firstly, the relationship between the variables was assessed to compute missing values and afterwards, the association between the estimators representing the genetic expression of bone biomarkers and bone mineral density was estimated. The final model, validated with bootstrapping as well, accounted for three demographic variables (sex, rheumatoid arthritis and corticoid use) and six variables of genetic expression of bone biomarkers (osteocalcin, Dkk2, sFRP1, Lrp6, WIF1 and Sema3A) and had an R-square of 0.967. The most prominent results achieved with this dissertation are related to the findings in model 3 that demonstrate that an upregulation of the Wnt regulators is associated with low bone mass in elderly Portuguese men and women.

To conclude, the developed work showed promising results related to the estimation of bone mineral density based on its association with the serum levels and the genetic expression of bone turnover biomarkers. It opened great study opportunities for future projects that may contribute to the inclusion of more bone biomarkers and the discovery of new associations and better bone mineral density estimates. Moreover, these types of models can, eventually, become a new clinical decision support system and help to reduce the frequency of DEXA exams prescribed to diagnose osteoporosis.

6.2 Future Perspectives

The project here developed showed promising estimates of bone mineral density based on its association with serum levels and genetic expression of bone biomarkers. However, there is still a wide range of complementary studies and improvements that must be conducted so that it can eventually pass from an investigation level to a clinical level.

Taking into consideration the results obtained in models 2.2 and 3, it would be of interest to study such relationships in a much larger and more complete clinical database, to ensure that more robust conclusions can be drawn from the models.

Studying the association between bone mineral density and bone biomarkers in men and women separately would also be of interest, since the concentrations of bone biomarkers, as well as their genetic expression, is different between sexes. Moreover, the inclusion of other study variables, such as calcium, growth factors and bone morphogenetic proteins may create more robust models, in a way that they can be more complete and better represent the complex bone microenvironment.

To finalize, it is of my belief that the present project has created several opportunities for future investigation work, hoping that the conclusions and reflections here presented will contribute to the development of enhanced models, bringing them closer to a clinical decision support system tool.

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Attachments

1. Calculation of the mean percent error of model 1

$$Mean\ Percent\ Error = \frac{|BMD\ DEXA - Estimated\ BMD|}{BMD\ DEXA} \times 100$$

2. Failed Approaches (*i.e.* all tested scenarios before reaching the final models)

Relative to model 1

- Inclusion of only women with DEXA measured BMD data;
- Inclusion of alcohol as a dependent variable;
- Dividing women by the reason of surgery.

Relative to model 2

- Removing all values that were outside the 90-percentile range;
- Normalize data with max and max-min techniques;
- Using only women as population-target;
- Pearson correlations between dependent variables;
- Using the median of the dependent variables to compute the missing values for the respective variables;
- Using generalized linear and non-linear models instead of linear regression;
- Inclusion of the regression constant;
- Dividing women by the reason of surgery.

Relative to model 3

- Removing all values that were outside the 90-percentile range;
- Normalize data with max and max-min techniques;
- Using only women as population-target;
- Pearson correlations between dependent variables;

- Using the median of the dependent variables to compute the missing values for the respective variables;
- Using generalized linear and non-linear models instead of linear regression;
- Inclusion of the regression constant;
- Dividing women by the reason of surgery.