Cross-sectional and longitudinal study of bone mineral status in older men.

Stefan Goemaere, MD

Thesis submitted for the fulfillment of the requirements of the degree of
Doctor in Health Sciences
by Stefan Goemaere, MD

Promotor: Prof. Dr. Jean-Marc Kaufman, MD, PhD

Co-promotor: Prof. Dr. Gust verbruggen, MD, PhD

UNIVERSITEIT GENT

Faculteit Geneeskunde en Gezondheidswetenschappen

Promotor
Prof. Dr. J.M. Kaufman
Ghent University

Co-Promotor
Prof. Dr. G. Verbruggen
Ghent University

Dean
Prof. Dr. G. Vanderstraeten
Ghent University

Rector
Prof. Dr. A. Depaepe
Ghent University

Commission for Examination

Prof. Dr. G. Vanderstraeten
Ghent university, guy.vanderstraeten@ugent.be

Prof. Dr. JY Reginster
University of Liège, jyreginster@ulg.ac.be

Prof. Dr. R. Adler
McGuire Veterans Affairs Medical Center, Richmond, USA, Robert.Adler@va.gov

Prof. Dr. Guy T'Sjoen
Ghent University, guy.tsjoen@UGent.be

Prof. Dr. Mirko Petrovic
Ghent University, mirko.petrovic@UGent.be

Prof. Dr. Jean De Schepper
Ghent University, jean.deschepper@uzgent.be

Prof. Dr. Jan Victor
Ghent University, jan.victor@uzgent.be

Only doubt is certain and disbelief worth believing.
Without this courage there can be no learning.
Believe nothing !

Anonymous

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Chapter 1 Introduction

1 General introduction: Bone Biochemistry, Bone Biology and Bone Quantity and Quality Assessment – An Overview

1.1 The skeletal function and anatomy

1.1.1 Introduction

The skeletal system is comprised of specialized connective tissues, namely bone and cartilage. The primary functions of the skeleton are 1) mechanical: to allow locomotion by providing a rigid support and sites for muscle action; 2) protective: to provide a shield for vital organs and bone marrow; 3) metabolic : to serve as a reservoir of growth factors and ions, in particular calcium and phosphate, for maintenance of the serum mineral homeostasis; and 4) endocrine: to secrete compounds for systemic regulatory action. In addition, the bone marrow serves as a source of precursor cells of the hematopoetic and mesenchymal lineage.
Compared to other tissues, bone is unique in its capacity of self-renewal and repair without scare tissue. Recommended readings for the following paragraphs on the bone anatomy are: *Turner CH & Burr DB, 1993; Eriksen EF et al, 1994; Simon SR, 1994; Burr DB et al, 1997; Ducy P et al, 2000a; Rodan GA & Martin TJ, 2000; Teitelbaum SL, 2000a&b; Manolagas SC, 2000a; Khosla S, 2001a; Marcus R, 2001; Bilezikian JP et al, 2002; Burr DB, 2002; Bouxsein ML, 2003.*

1.1.2 Bone anatomy and macroscopic organisation

The human skeleton is a complex organ consisting of 206 bones (126 appendicular, 74 axial and 6 ossicles). There are two major types of bones in the skeleton: flat bone (i.e.; skull, scapula, mandibula and ilium) and long bones (i.e. radius, tibia, femur, humerus etc …). These two types of bone, flat versus long bones, are distinguished by their mechanism of development, respectively intramembranous versus endochondral ossification and by their locations in the body, i.e. in axial (or central) versus appendicular (or peripheral) skeleton.

The long bone **(Figure 1)** shows a hollow tube in the center, the diaphysis, with wider regions at each end, the epiphysis, and a transition / developmental zone in between, the metaphysis. The outer surface of the epiphysis forms the joint surface and is covered by a layer of articular cartilage. In the growing long bone the epiphysis and metaphysis are separated by a layer of cartilage, namely the growth plate. This distinctly structured region is responsable for the longitudinal bone growth.

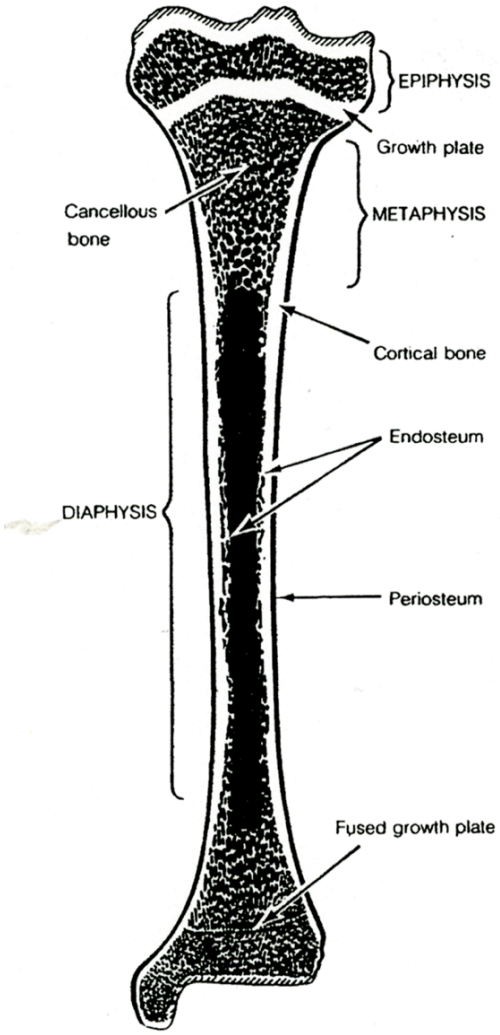


Figure 1: Schematic view of a longitudinal section through a growing long bone (Reproduced from Bouxsein ML 2006, Advanced Osteoporosis Course; IOF).

The external surface of bone is formed by a thick and dense layer of calcified tissue, termed the cortex: also called cortical or compact bone. In the diaphysis, the cortex surrounds the medullary canal where the hematopoetic bone marrow is located. Towards the metaphysis and epiphysis, the cortex becomes progressively thinner as it surrounds a network of rods and plates (trabeculae), termed trabecular or cancellous bone.
Trabecular and cortical bone are distinguished by their structure, particularly in the fraction of their volume that is mineralized. In healthy bone, over 85% of the cortical bone volume is mineralized (i.e. the porosity less than 15%), whereas only 20-40% of the volume of the trabecular bone is mineralized, the remainder being occupied by bone marrow, blood vessels and other connective tissue. Overall cortical and trabecular bone comprise approximately 80% and 20%, respectively, of the total mass of the skeleton. Due to its structure, trabecular bone has a much larger surface area exposed to marrow and soft tissues than cortical bone. As bone resorption and formation occur on bone surfaces, cancellous bone is approximately 6-8 times more metabolically active than cortical bone. As a result, changes in bone due to age, diet, disease and pharmacological intervention occur sooner and are more pronounced at the cancellous compared to the cortical bone.

Functionally, cortical bone fulfills more a structural function (mechanical & protective) whereas trabecular bone serves a metabolic function, although it is also essential for transmitting loads from the joint surface to the cortex.

There are two surfaces where bone is in contact with soft tissues: an external surface (the periosteal surface) and an internal surface (the endosteal surface). These two surfaces are lined with organized layers of cells, termed the periosteum and endosteum, respectively. The endosteal compartment can be divided in endocortical and trabecular surfaces. The local bone cell microenvironment and biochemical milieu associated with each bone surface influences its response to osteogenic and osteoclastic stimuli.

1.2 Bone histology: microscopic structure and hierarchial organisation

Bone is a complex living material having a hierarchial structure **(Figure 2)** *(Rho JY et al, 1998)* . The mechanical behavior depends on an interaction of the properties of each level of this structural hierarchy. The basic composition of bone, like all connective tissues, consists of cells and extracellular matrix. Bone tissue is composed of a mineral phase in an organic matrix *(Boskey AL, 2003)*. The extracelluar matrix is mainly comprised of collagen, but also of noncollagenous proteins and proteoglycans retaining water.

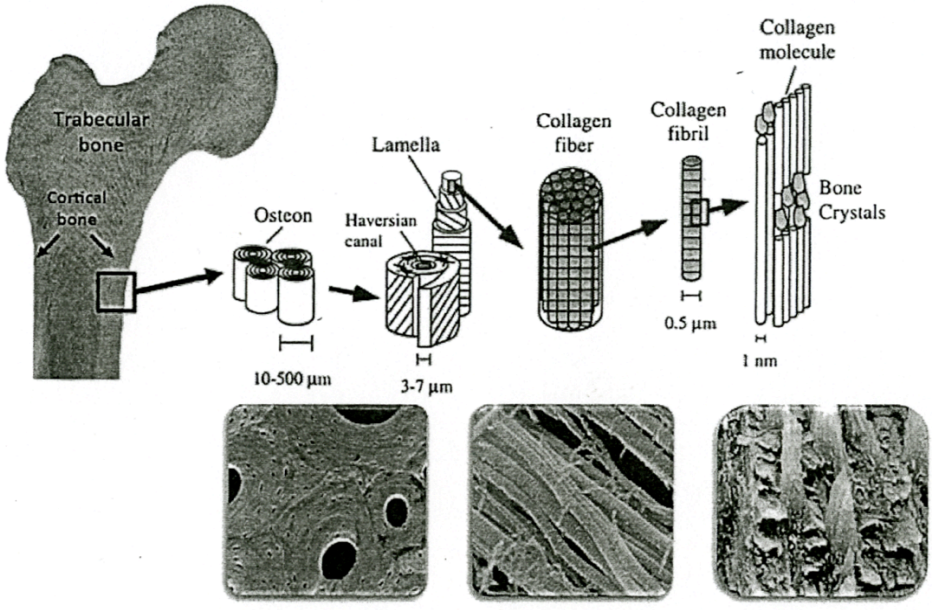


Figure 2: Hierachical structure of bone for the macro- (left panel) to the sub-nanostructure (right panel) and related in situ micrographs using electronic microscopy (Adapted from Rho JY et al, 1998)
The specific composition of bone varies by skeletal sites depending on its function, the age, diet and disease conditions. In general, the mineral or inorganic phase accounts of 60-70% of the tissue. Water accounts of 5-10% and the organic components comprise the remainder. The calcified matrix is not inert, as cells (osteocytes) are embedded in the matrix and are playing a critical role in the local activation of bone remodeling (see below)

At the tissue level, bone is composed of bone structural units (BSU) – the bone packets in trabecular bone **(Figure 3)** and the osteons in cortical bone **(Figure 4)**. A BSU consists of the net production of bone tissue following a remodeling cycle: bone remodeling unit (BMU). The different phases of a bone remodeling cycle occur in a well-organized manner. In healthy adult conditions, after initiation of a cycle bone resorption and bone formation perfectly follow eachother in a specific time sequence and in a perfectly balanced way in respect to the amount of bone remodeled and the bone site considered. The molecular and cellular regulation of this coupled and balanced process is not yet completely unraveled, but the most recent findings are described below.

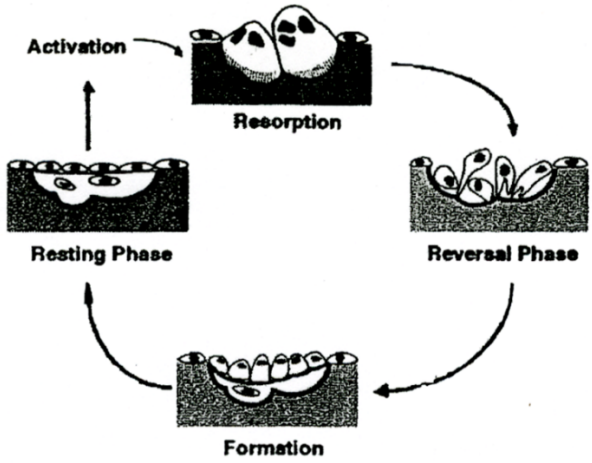


Figure 3: Bone Structural Unit (BSU) (bone packet) and Bone Remodeling Unit (BMU) in the trabecular bone. (Reproduced from Bouxsein ML, 2006, Advanced Osteoporosis Course; IOF)

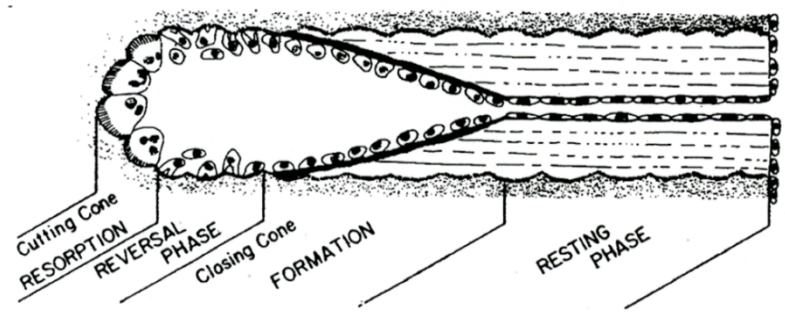


Figure 4: Bone structural unit (BSU) (osteon) and bone remodeling unit (BMU) in the cortical bone. (Reproduced from Bouxsein ML, 2006; Advanced Osteoporosis Course; IOF)

1.3 Bone Matrix

Bone forming cells synthesize an extracellular matrix. This bone extracellular matrix is made up of inorganic and organic components which account for 60 and 40% of its dry weight, respectively. The organic components include collagen and non-collagenous proteins, proteoglycans, cytokines and a number of growth- and differentiation factors.

1.3.1 Bone collagens

Aproximately 90% of the organic component of the extracellular matrix is type I collagen. Collagen type I fibers provide tensile strength to the tissue and serve as a substrate for mineralization. Collagen is a protein consisting of three polypeptide chains containing approximately 1000 amino acids each. Type I collagen is formed by a triple helix of two indentical α1(I) chains and a unique α2 chain stabilized by cross-links between chains **(Figure 5)**.

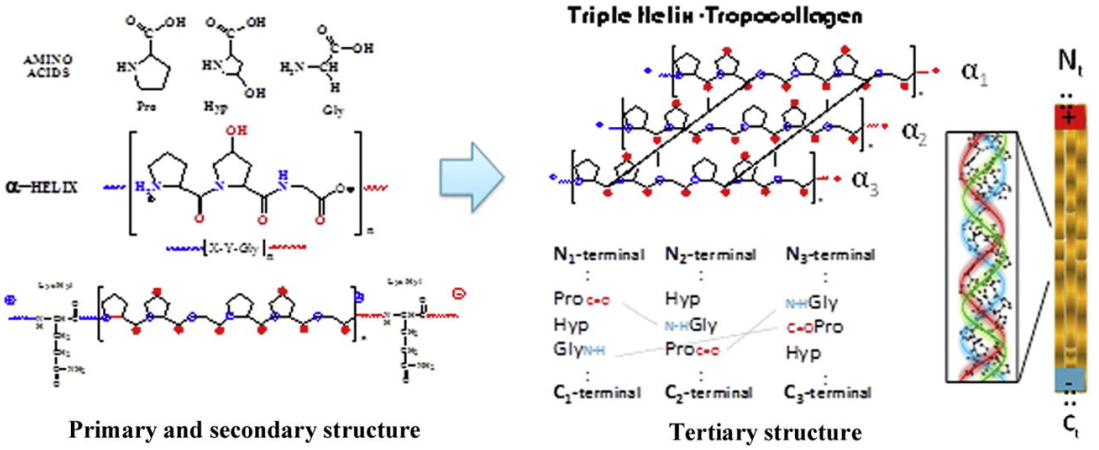


Figure 5: Primary, secondary and tertiary structure of bone collagen.

Each collagen molecule is aligned paralel with the next to form a collagen fibril, and arranged in a staggered fashion that leaves "holes" or gaps which are the sites for mineral deposition and cristal formation **(Figure 2)**. In general, collagen fibers and mineral crystals have a preferential orientation, which alternates in adult bone from layer to layer, giving bone its typical lamellar structure, which is best seen under polarized light. This fiber orientation allows the highest density of collagen per volume of tissue. These lamellae are usually parallel to each other when bone is deposited along a relatively flat surface, such as the periosteum of trabecular bone or concentric if bone is deposited on a surface that surronds a blood vessel, i.e a Harvesian system or osteon, such as occurs in cortical bone.
Thus, in cortical bone the tissue has mainly an osteonal structure, where an osteon is comprised of a central nutrient blood vessel surrounded by concentric lamellae: in total, approximately 150-400 μm in diameter and up to 2 mm in length. The various osteons are in contact with each other by a complex network of osteocytic cell processes (see below), which allows for transfer of nutrients and mechanical signals from the cortical bone surfaces.
If bone is formed very rapidly, as is the case during growth, there is no preferential orientation of the collagen fibers. This type of bone, in which collagen fibers are loosely packed and randomly oriented, is called woven bone. Woven bone is essentially absent from the adult skeleton, except under pathological conditions such as fracture healing, Paget's disease or fluorosis.

1.3.2 Non-collagenous proteins (NCPs) of bone

Matrix non-collagenous proteins include 3 main types: osteocalcin, osteonectin and osteopontin. These proteins promote bone formation and mineralisation.

1.3.2.1 Osteocalcin

Osteocalcin (OC) is the most abundant of the non-collagenous bone matrix proteins and make up 10-20% of the bone protein content. Osteocalcin is produced by mature osteoblasts. The production of OC is stimulated by $1,25(OH)_2$ vitamin D3 and inhibited by parathyroid hormone (PTH). In a vitamin K dependent way OC is carboxylated (cOC) as a posttranslational modification and this form promotes mineralisation and formation of bone. The protein also attracts osteoclasts and thus effectively regulates bone density. Osteocalcin is detectable in the serum and urine and serves as a marker of bone formation (see below).
The undercarboxylated form of osteoblast-specific secreted osteocalcin (unOC) is a hormone favoring glucose metabolism and increasing energy expenditure in mice *(Karsenty G & Ferron M, 2012)*.

Communication from metabolism to bone was previously considered purely unidirectional, involving interactions among an adipocyte-derived factor (leptin) the sympathetic nervous system and neuropeptides. The new animal findings suggest that the skeleton may act as an endocrine tissue that regulates metabolic homeostasis (Lieben L et al, 2009).
However, human data are still inconclusive and more study is required to define the role of the carboxylated and undercarboxylate OC form in the regulation of glucose metabolism.

1.3.2.2 Osteonectin

Osteonectin is a 32.000 dalton bone-specific protein that binds selectively to both hydroxyapatite and collagen. When osteonectin is bound to insolubilized type I collagen, the resultant complex binds synthetic apatite crystals and free calcium ions. The osteonectin-collagen complexes also initiate mineral phase deposition from metastable balanced salt solutions. Osteonectin is a bone tissue-specific protein, linking the bone mineral and collagen phases, perhaps initiating active mineralization in normal skeletal tissue *(Termine JD et al, 1981)*.

1.3.2.3 Osteopontin

Osteopontin (OPN) is a highly phosphorylated sialoprotein that is a prominent component of the mineralized extracellular matrices of bones and teeth. OPN is a multifunctional protein, and although highly expressed in bone, it is also expressed by various cell types including macrophages, endothelial cells, smooth muscle cells and epithelial cells. OPN is involved in diverse biological processes *(Sodek J et al, 2000)*.
Expression of OPN in a variety of tissues indicates a multiplicity of functions, but the lack of a clear phenotype in OPN "knockout" mice has not established a definitive role for OPN in any tissue. Recent studies have provided some novel and intriguing insights into the versatility of this protein in diverse biological events, including developmental processes, wound healing, immunological responses, tumorigenesis, bone resorption and calcification. The ability of OPN to stimulate cell activity through multiple receptors linked to several interactive signaling pathways can account for much of the functional diversity **(Figure 6)**.

OPN is intimately involved in the regulation of both physiological and pathological mineralization. In normal bone tissue, OPN is expressed by both osteoclasts and osteoblasts which are the cells responsible for bone remodelling. During normal bone mineralization, osteoclast-derived OPN inhibits the formation of hydroxyapatite.

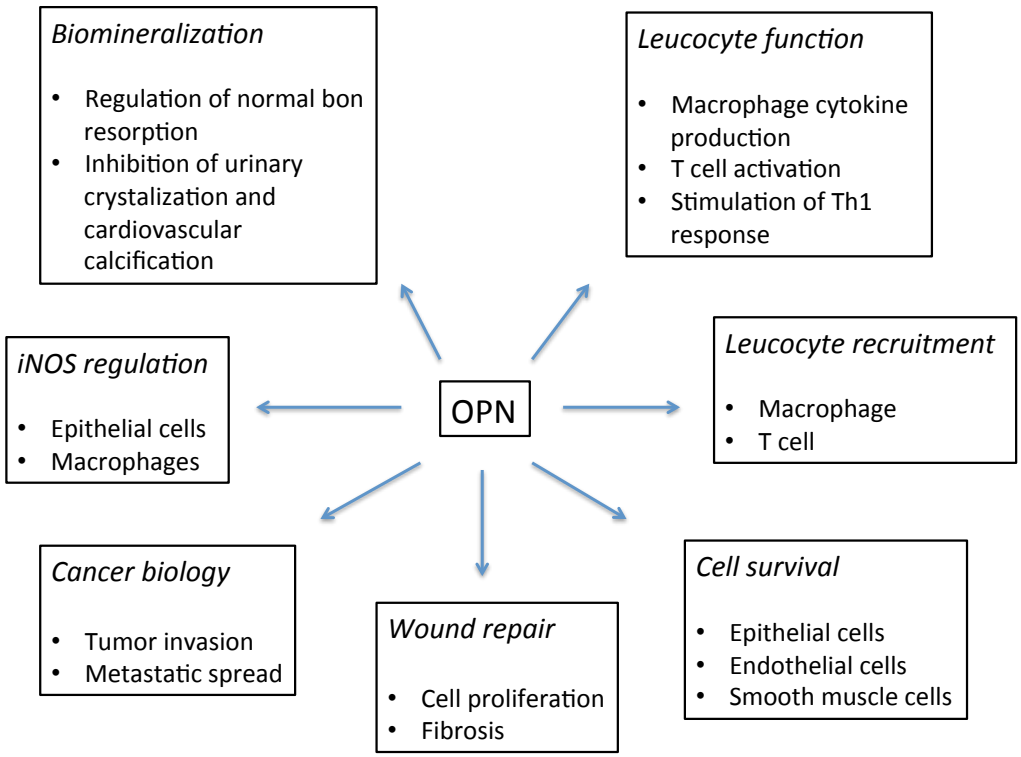


Figure 6 : The various important putative biological functions of osteopontin (OPN)

1.3.3 Proteoglycans of bone

The different forms and the pattern of localisation of proteoglycans (PGs) in bone reflect their important role in the organisation of the bone extracellular matrix.
Osteoblasts produce aggrecan and decorin. Chondroitin-6-sulfate is the main glycosaminoglycan in these proteoglycans *(Mania VM et al, 2009)*. The presence of chondroitin-4-sulfate and of traces of hyaluronan has also been reported *(Engfeldt B & Hjerpe A, 1976)*.

Proteoglycans are a ubiquitous family of biomolecules that are composed of a core protein and one or more covalently attached sulfated glycosaminoglycans (GAG) chains. PGs have been found associated with intracellular compartments, the cell surface, the extracellular matrix and basement membranes in almost all tissues in adults. GASs are linear polymers of repeated discaharidic units of uronic (glucoronic or iduronic) acid and N-acetylated hexosamine (glucosamine or galactosamine), except for keratan sulfate (KS), which is composed of galactose that can be modified (sulfation position) at irregular intervals along the chain. The glucosaminoglycans (heparin (HP), heparin sulfate (HS), keratan sulfate (KS)) and the galactosaminoglycans are shown in **Figure 7**. The degree and position of sulfate as well as the degree and position of 5' epimerisation are extremely variable depending on the tissular/cellular/metabolic context *(Hardingham TE & Fosang AJ, 1992)*.
Hyaluronic acid is a non-sulfated, non-attached to protein GAG composed of D-glucuronic acid and D-glucosamine **(Figure 7)**.

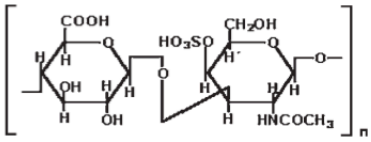
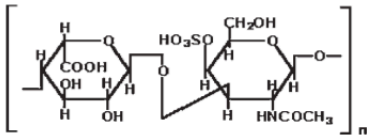
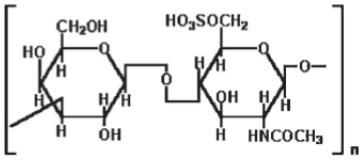
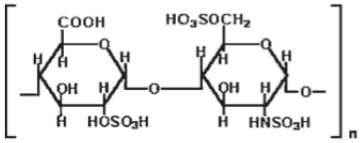
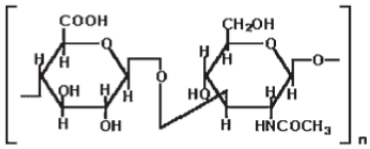
Repeating disaccharide of GAG	Sulfation position
 **chondroitin sulfate** (glucuronic acid + N-acetylgalactosamine)	$GalNAc\text{-}4SO_4$ $GalNAc\text{-}6SO_4$ $GalNAc\text{-}4,6SO_4$
 **dermatan sulfate** (glucuronic acid or iduronic acid + N-acetylgalactosamine)	$GalNAc\text{-}4SO_4$ $GalNAc\text{-}6SO_4$ $(IdoA\text{-}2SO_4)$
 **keratan sulfate** (galactose + N-acetylglucosamine)	$GlcNAc\text{-}6SO_4$ $Gal\text{-}6SO_4$
 **heparin and heparan sulfate** (glucuronic acid or iduronic acid + N-acetylglucosamine)	$(IdoA\text{-}2SO_4)$ $GlcNAc\text{-}3,6SO_4$ $(GlcA\text{-}2SO_4)$
 **hyaluronic acid** (glucuronic acid + N-acetylglucosamine)	no sulfation

Figure 7: Disacharide composition of the different glycosaminoglycan families.
(Reproduced from Lamoureux F et al, 2007)

There is no unifying feature for core protein structures in proteoglycans as they display a great diversity of protein forms. This huge variability in protein structure provides specific functions of the different PG families, including among others small leucine-rich repeat PG (SLRPs), heparan sulfate PG and aggrecan **(Figure 8)**.

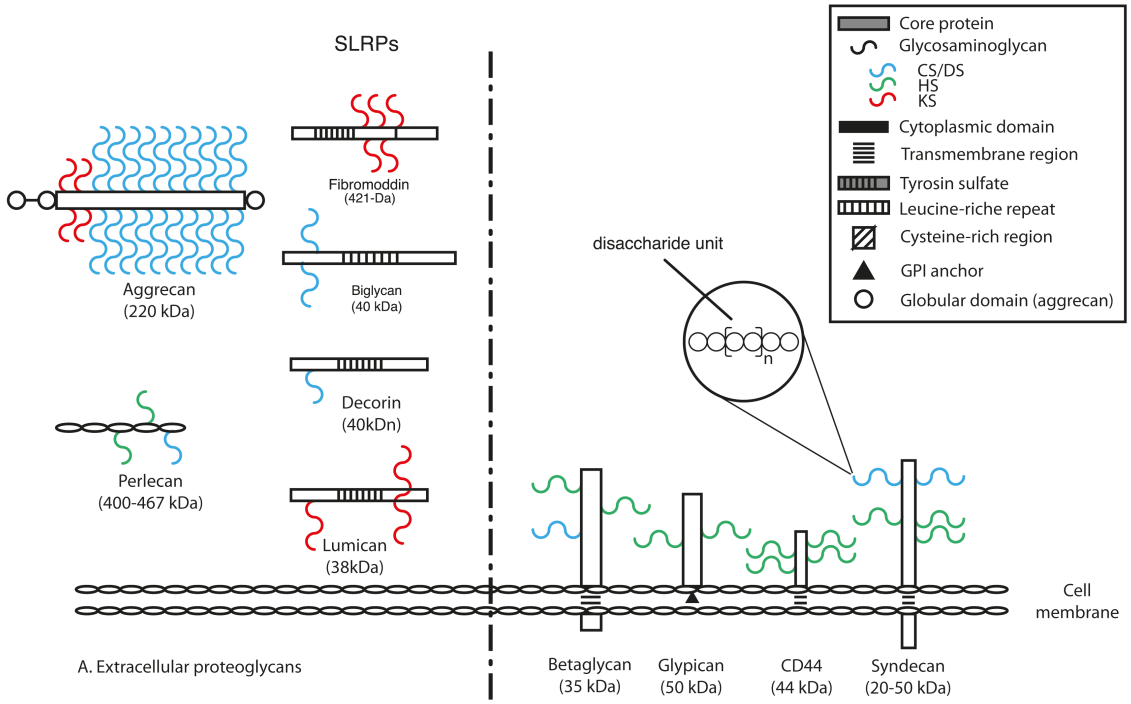


*Figure 8 : Bone proteoglycan classification and structure : **A:** extracellular proteoglycan can be broadly classified as small leucine rich-repeat proteoglycan (SLRPs), chondroitine sulfate containing PGs (aggrecan) and heparin sulphate-containing proteoglycan (perlecan). **B:** Membrane-associated proteoglycans are classified as transmembrane proteins (syndecan family, CD44, betaglycan) or glycosyl-phosphate inositol-anchored proteins (glypicans). The high variability of PG structure can be evaluated by the difference in core protein size (as indicated) and different compositions of GAG chains (blue: CS/DS chains; green: HS chains; red: KS chains). (Reproduced from Lamoureux F et al, 2007).*

Bone proteoglycans, through their compositional diversity, play a broad role in this specific tissue: (1) they act as crucial structural elements in the supramolecular organisation of other extracellular matrix macromolecules, e.g. the fibrillogenesis of the collagen fibers; (2) they act as co-receptors for cytokines, several growth and differentiation factors (e.g. TGFβ) and osteoprotegerin (OPG), playing important role in their localization, thereby regulating their bioavailability and activity **(Figure 9)**; (3) they take part in signalling mechanisms that govern bone formation, resorption and homeostasis **(Figure 10)**; (4) they account for the compressive strength of the tissue and inhibit mineralisation.
The function of distinct bone proteoglycans is illustrated in a series of knockout mice with phenotypes in bone and cartilage *(Lamoureux F et al, 2007).*

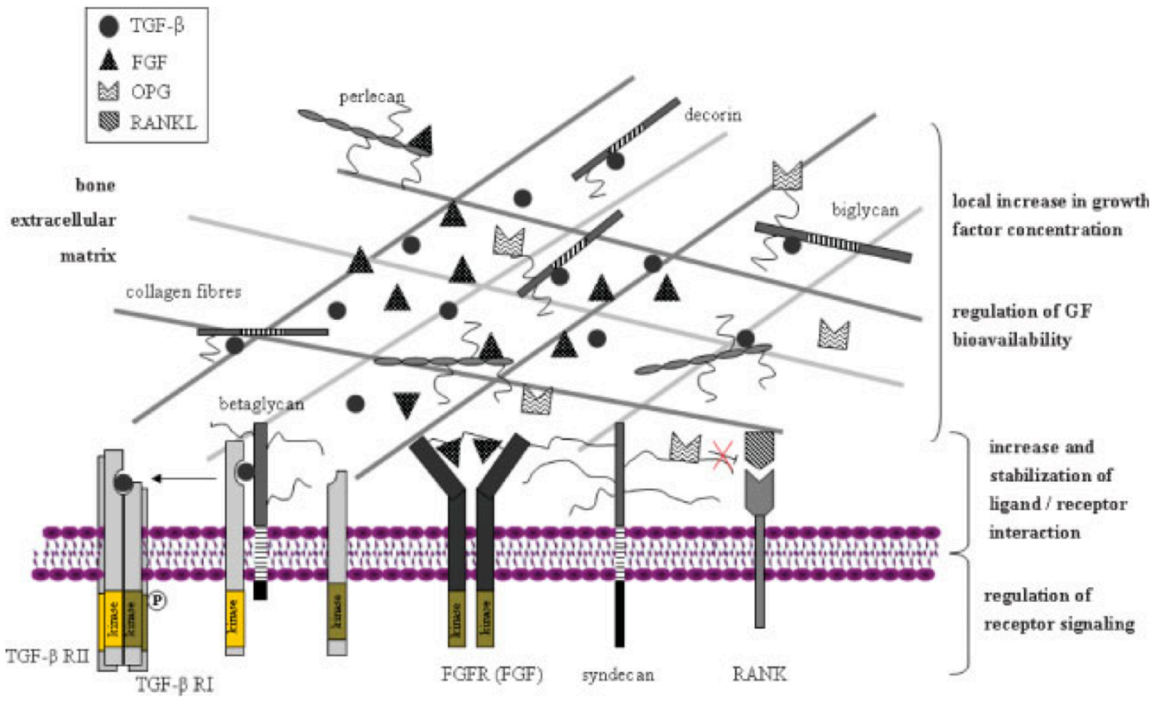


Figure 9 : Proteoglycans as regulators of growth factor activity in the bone. Schematic representation of interactions of proteoglycans present in bone (from both extracellular (perlecan, decorin, biglycan) and periarticular (betaglycan, syndecans) compartments with bone regulating growth factors such as transforming growth factor-β (TGF-$_{β}$), osteoprotegerin (OPG) or fibroblast growth factor (FGF). These growth factors can be stored in the bone extracellular matrix (ECM), by binding to decorin, biglycan or perlecan leading to local increase in growth factor concentration and/or regulation of growth factor availability. Membrane-bound PG such as syndecan or betaglycan may act as co-receptors for these growth factors, forming multimolecular complexes with FGF-FGFR (syndecans), RANK-RANKL-OPG (syndecans) or TbR1/-TbR2: TGF-b (betaglycan), leading to increase and stabilization of ligand/receptor interactions and regulation of receptor signaling *(Reproduced from Lamoureux F et al, 2007).*

1.3.3.1 Small leucine-rich repeat proteoglycans (SLRPs)

This class of PGs represents the most-abundant kind of PG in the bone matrix **(Figure 8)**. Decorin, biglycan (BGN), fibromodulin, lumican, osteoadherin and PG-Lb have been well decribed in bone tissue. The core protein of the PGs are proposed to adopt a horseshoe structure that may facilitate protein-protein interactions with a range of other matrix components (mainly collagen fibres) and with the mineral phase during the formation of calcified tissues **(Figure 9)**.

Decorin and biglycan in mineralized matrices have been substituted with chondroitin sulfate predominantly, wheras in soft tissues they mostly carry dermatan sulfate.
The small keratan sulfate PG fibromodulin is deposited pericellularly around the late-hypertrophic chondrocyte of the secondary ossification center and in the growth plate and is suggested to play a role in the endochrondral ossification *(Alini M & Roughly PJ, 2001; Saamanen AM et al, 2001)*.
Lumican, a fibril associated molecule is implicated in regulating the progression of fibril assembly *(Ezura Y et al, 2000)*.
Osteoadherin, a bone PG containing keratan sulfate was detected in mature osteoblasts located on trabecular bone. It is exclusively identified in primary bone spongiosa *(Wendel M et al, 1998)*.
PG-Lb is a chondroitine/dermatan sulfate PG suggested to participate in the osteogenic processes *(Shinomura T et al, 1992)*.

Numerous studies have demonstrated that SLRPs are involved in the structural organisation of the bone matrix. Understanding of their functions has been obtained by multiple studies in vivo in mice deficient in one or two of the four most abundant SLRPs (biglycan, decorin, fibromodulin and lumican).
Apart from their involment in the structural organisation of the bone matrix, SLRPs play an essential role in the regulation of growth factors, among them TGF-β. High concentrations of TGF-β are found in the bone matrix, reflecting a pivotal role of this factor in the coupling of bone resorption and formation. TGF-β binding experiments have shown that the growth factor was nearly exclusively bound to decorin, via its core protein *(Takeuchi Y et al, 1994)*. This binding increases TGF-β binding to its receptor and enhances its bioactivity.

1.3.3.2 Hyaluronan/CD44, aggrecan and chondroitine sulfate containing proteoglycans.

The expression of aggrecan is not restricted to cartilage. They may be expressed in certain conditions by osteoblasts *(Wong M, 1992)*. Hyaluronan is observed in regional areas of the periosteum and endosteum. CD44, a cell surface hyaluronan receptor was identified on osteoclasts, chondrocytes with anabolic and catabolic function, osteocytes and hematopoietic marrow cells *(Noonan KJ et al, 1996)*.

1.3.3.3 Heparan sulfate proteoglycans (HSPG)

Immunohistochemical studies of HSPG localization suggest that osteblast and osteocyte synthesize HSPG and both membrane and matrix HSPG are localized in bone tissue. HSPG may play an important role in cell-cell interactions between fibroblast-like cells and osteoclast lineage cells by heparin-binding growth factors and/or heparin binding adhesion molecules such as fibronectin *(Nakamura H & Ozawa H, 1994)*.

1.3.3.4 Minor bone proteoglycans

Several extracellular PGs do not belong to any family. Their core protein has common properties with the bone sialoprotein II molecule that do not possess GAG chains. Their role is not well defined currently.

1.3.3.5 Regulation of bone resorption by proteoglycans

An emerging role of PGs in bone biology is the control of osteoclast activity and bone resorption **(Figure 10)**. As shown below, the molecular triad OPG-RANKL-RANK orchestrates the physiological bone remodeling and represents the final effectors of bone resorption regulation.

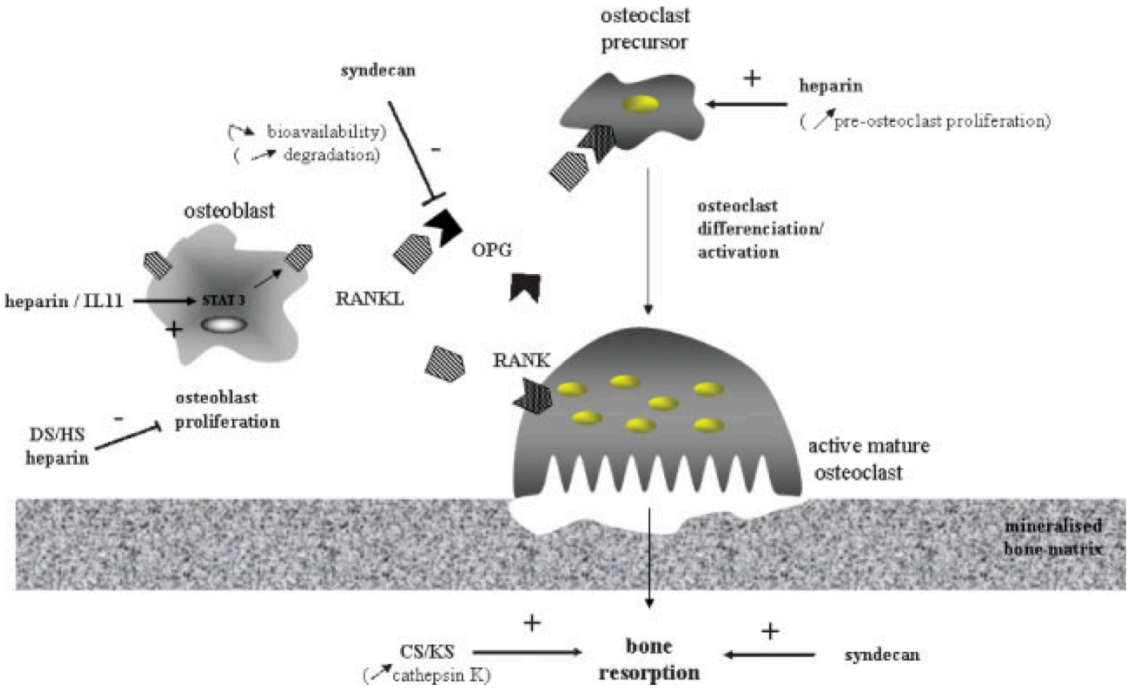


Figure 10: PGs act as regulators of bone remodeling by interfering with osteoblast and osteoclast biological activities. Glycosaminoglycans (GAGs) or PGs mainly act as pro-resorptive agents: dermatan sulfate (DS), heparin sulfate (HS) and heparin diminish osteoblast proliferation and heparin associated with interleukin-11 (IL-11) enhance RANKL expression via STAT3, syndecan-1 inhibits osteoprotegerin (OPG) bioavailability, heparin enhances pre-osteoblast proliferation, syndecan activates bone resorption and chondroitin sulfate (CS)/keratin sulfate (KS) enchance cathepsine K activity. All these effects lead to osteoclast differentiation and activation, the to bone resorption. (Reproduced from Lamoureux F et al, 2007).

Osteoprotegerin (OPG), a protein belonging to the tumor necrosis factor (TNF) receptor family contains three structural domains specifically influencing its biologic activities. One of these is a heparin-binding domain potentially capable of interacting with PGs *(Simonet WS et al, 1997)*. Kinetic data demonstrated that OPG binds to heparin with high affinity and that preincubation of OPG with heparin inhibited, in a dose dependent manner, the OPG binding to the RANK-RANKL complex. GAGs from different structure/origin (HS, DS, and CS) exert similar activity on OPG binding. Sulfation is essential in the OPG-blocking function of GAGs. Therefore GAGs are able to diminish OPG availability and bioactivity and so indirectly increase bone resorption through the inhibition of interaction with RANKL.
HSPG can also participate in bone resorption regulation through the modulation of cathepsin K activity. Cathepsin K, a lysosomal papain-like cysteine protease mainly involved in bone matrix destruction, forms complexes with chondroitin sulfate and demonstrates a highly collagenolytic activity.

1.3.4 Bone mineralisation

The inorganic component of bone is largely comprised of calcium phosphate mineral analogous to crystalline calcium hydroxylapatite [$Ca_{10}(PO_4)_6(OH)_2$], though it is not pure and may contain other constituents, such as magnesium, potasium, strontium, sodium, carbonate and fluoride. Bone mineral is a reservoir of ions that can be stored and released during remodeling to maintain phosphocalcic equilibrium.
The degree of mineralization of bone (bone density measured at the tissue level) and the characteristics of the mineral deposited are major determinants of bone strength. The apatite is present as a plate-like crystal approximately 20-80 nm long and 25 nm thick. Newly formed bone is less mineralized and has smaller crystal size than older, more mature bone. Having either smaller than average or larger than average mineral crystals impairs bone biomechanical properties.

In comparison with matrix formation, which is determined by the number and activity of osteoblasts, matrix mineralization is a multistep, autonomous, passive process that occurs in three stages: primary and secondary mineralization and maximal mineralization. The matrix achieves approximately 70-80% of its full mineralisation level relatively rapidly by its initial deposistion, i.e. within months, called the primary mineralization. Further mineralization maturation up to the highest mineral density in a given volume of matrix proceeds more slowly. So matrix may take years or decades to achieve its maximal mineralization. The latter includes an increase in number, size and perfection of crystals.

The rate of bone turnover determines largely the extent of secondary mineralization, and thus in situations of increased bone turnover, e.g. recent menopause, bone is relatively undermineralized, whereas in conditions of low bone turnover, e.g. under antiresorptive therapy, the degree of matrix mineralization increases.
Bone remodeling occurs asynchronously in various regions of a given bone. This results in a heterogeneous distribution of mineralization throughout bone tissue. The degree of mineralization in each BSU is thus dependent of the time that elapsed since its deposition *(Boivin G & Meunier JP, 2002; Roschger P et al, 2008)*. The distribution and degree of mineralization at the tissue level of cortical and trabecular bone is tightly related to (1) the activation frequency (Ac.F) of bone remodeling, which can be influenced by age, hormonal status, pathologies, or therapy *(Boivin G & Meunier JP, 2002; Roschger P et al, 2008;)*; (2) the balance between bone formation and bone resorption and (3) the kinetic of primary and secondary mineralization.
The bone remodeling activity, as a regulator of the degree of bone mineralization (DBM) and heterogeneous distribution of the mineral at the tissue level, is directly affecting bone mechanical properties. The mean DBM is positively associated with stiffness at both BSU and organ level *(Follet H et al, 2004; Bala Y et al, 2011).* At the organ level, as the stiffness increase, the work to failure decreases *(Currey JD, 2006)*. Therefore, an increase in DBM is not necessarily synonymous to higher bone strength. The energy required for a crack to propagate will be the lowest in a homogeneous and more mineralized bone tissue *(Ciarelli TE et al, 2003; Currey JD, 2006; Boskey AL et al, 2009; Renders GA et al, 2011)*.

However, processes at the nanostructural level may be independent of bone turnover. A more global comprehesion is warranted of the effects of bone quality characteristics, resulting from changes at the nano- or micro-structural levels, on bone biomechanical properties. These can be considered at either the local (BSU) or global (tissue and organ) level *(Yerramshetty JS & Akkus O, 2008)*. Independently of bone mass and its distribution in space, the mineralization and the "quality" of the mineral play a crucial role in the elastic, plastic and viscoelastic properties defining the mechanical behavior of bones *(Ferguson VL et al, 2009; Bala Y et al, 2011)*.

1.3.4.1 Physiology of bone mineralisation process

Human bone mineral is composed of a poorly crystalized, Ca-deficient and nonstoichiometric apatite. It contains major elements, such as Ca^{2+} (40 wt %), PO_4^{3-} (18 wt %), CO_3^{2-} (6-7wt %), minor elements: i.e. Mg^{2+} or Na^{+}, and trace elements: Sr^{2+}, F^{-} and others as a function of environmental composition.
Bone crystals can be depicted in three compartments **(Figure 11)**: (1) apatitic core; (2) hydrated layer and (3) surrounding fluid. Ions in the hydrated layer are very labile and reactive. They are easily and reversibly exchangeable. During mineral maturation, the decrease in labile nonapatitic environments is associated with an increase in stable apatitic environment *(Cazalbou S et al, 2004; Boivin G, 2007;).*
The ability to exchange ions with the fluids allows the initial formation, growth, maturation, and dissolution of crystals *(Boivin G, 2007).* The water level decreases as the space becomes occupied by the mineral phase. Ions no longer diffuse in the milieu, and crystal growth stops *(Fratzl P et al, 1993; Boivin G, 2007)*.

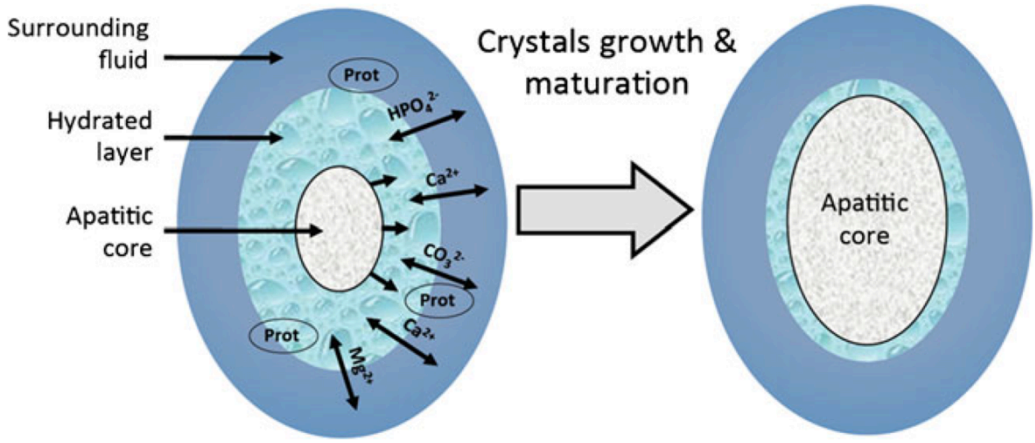


Figure 11: Evolution of the hydrated layer and apatite core during the growth and maturation of the crystal. The hydrated layer allowing ionic exchange between the apatite core and the surrounding fluid progressively decreased, leading to a stable apatitic domain. (Reproduced from Bala Y et al, 2013)

Mineral crystallites are nanosized platelet-shaped: 1-7 nm in thickness, 15-200 nm in length and 10-80 nm in width *(Fratzl P et al, 2004)*. Their organization in bone tissue depends on the structural properties of the organic matrix *(Riggs CM et al, 1993)*. The mineral crystals are arranged parallel to each other and oriented parallel to the longitudinal axis of the collagen fibrils in a staggered arrangement, with the first nucleation in the gap zones of the collagen fibrils **(Figure 2)**.

Regarding the impairments of bone strength occuring with pathologies leading to larger crystals (increase in brittleness) and smaller crystals (increase in ductility; ability of a material to deform plastically without breaking) or abnormal distributed crystal size, it is thought that an "optimal" size and heterogeneity determines a maximal strenght *(Boskey AL, 2003)*.
The presence of the apatite enhances the tensile modulus and strength of collagen. The inorganic matrix conversely, directly acts on the proportion of loads transferred on the mineral particles preventing mineral cracking.

1.3.4.2 Determinants and regulation of bone mineralization

1.3.4.2.1 Collagen and non-collagenous proteins

Crystal nucleation is triggered by collagen and noncollagenous proteins, which also regulate several steps of mineralization. The crystal first elongates in a plate-like shape, and then grows in thickness rather than in length *(Fratzl P et al, 1991).* The size of the crystals may be altered by extrinsic or intrinsic factors, including the properties of organic matrix, the tissular distribution of noncollagenous proteins acting in the formation and growth of the crystals, environment, diet, ageing, bone remodeling rate, pathologies and medications *(Boskey AL, 2003)*.
The quality of bone mineral can be described by its crystallinity, maturation, and level of substitution. Crystallinity encompasses the size and perfection of the crystal lattice *(Handschin RG & Stern WB, 1995; Farlay D et al, 2010;)*. The mineral maturity reflects the conversion of nonapatitic precursors into apatite mineral. During normal mineralization of bone, crystallinity and maturation usually evolve concomittantly and are well correlated.
Carbonate ions (CO_3^{2-}) present in bone can modify crystallinity. There is controversy about whether there is an increase *(Petra M, 2005)* or a decrease *(Paschalis EP et al, 1996)* of CO_3^{2-}/PO_4^{3-} ratio with mineral maturation.

1.3.4.2.2 Enzyms regulating bone mineralisation

Alkaline phosphatase (ALP) produced by the osteoblasts is an enzyme involved in mineralization of bone by cleavage of phosphate from organic phosphate. The data show that the initiation and progression of mineralization are separate phenomena. Organic phosphate and alkaline phosphatase play a crucial role in the initiation of mineralization but are not required for the continuation of mineralization of bone nodules *(Bellows CG et al, 1991)*.

1.4 Cell components of bone

Bone cells include lining cells, osteblasts, osteoclasts and osteocytes and their respective precursors. Each of these cells has an unique morphology and function. It is important that most of the bone turnover (or remodeling) occurs at the bone surface, primarily at the endosteal surface and intracortical haversian systems which interface with bone marrow. These surfaces display a heterogenous population of cells, reflecting specific cellular activities and stages of bone remodeling.

1.4.1 Osteoblasts

The osteoblast is responsible for bone formation, i.e. production of the bone matrix, including collagen and ground substance. Osteblasts are derived from mesenchymal stem cells : bone marrow stromal stem cells or connective tissue mesenchymal stem cells. Under appropriate stimulation, these osteoblast precursor cells undergo proliferation and differentiate into pre-osteoblasts and finally mature osteoblasts. Osteoblasts are found in clusters of cuboidal-shaped cells lining bone surface (100 to 400 cells per bone forming site) where they unidirectionally secrete an organic matrix (approximately 1 μm/day), namely osteoid or osteoid seams. Osteoid exist because there is a lag between the deposition of the matrix and the initial step of its extracellular mineralization, which occurs about 5 to 10 days later *(Bala Y et al, 2010)*. The osteoblasts produce alkaline phosphatase, but their role in the mineralization process is still not fully understood. When the osteoblast stops secreting matrix, it can become a lining cell or is embedded within the matrix, and can further differentiate into an osteocyte. Other osteoblasts undergo premature programmed cell death (apoptosis).

1.4.2 Osteocytes

Osteocytes, as matrix embedded osteoblasts, are located in osteocytic lacunae and are the most abundant cells in bone. Osteocytes have numerous, long cell processes rich in microfilaments that are in contact with the cell processes of other osteocytes or that of cells lining the bone surface. These cell processes form a complex interconnected network of thin canaliculi that permeate throughout the bone matrix. This network is allowing bone to respond to a variety of stimuli with resulting local activation of bone remodeling. Viability of ostecoytes has been implicated in the quality and mechanical integrity of bone. Between the osteocyte's membrane and the bone matrix lies a periosteocytic space, filled with extracellular fluid. Fluid flow due to mechanial loading and mineral ion exchange between this fluid and the bone surface play important mechanical and metabolic roles.
Osteocytes are increasingly recognized as multifunctional cells that help orchestrate bone and mineral metabolism. In addition to their putative role as detectors/transducers of mechanical strain in bone, osteocytes are a major source of sclerostin, a key inhibitor of Wnt signaling and bone formation, and appear to be important regulators of osteoclast function via their production of NF-kB ligand (RANKL) and osteoprotegerin (OPG). It was also long believed that osteocytes could remove or even replace mineralized bone around their lacunae and canaliculi. This concept of "osteocytic osteolysis" has recently been reconfirmed in a number of reviews *(Teti A & Zallone A, 2009; Bonewald LF et al, 2011; Atkins GJ & Findley DM, 2012)*. Numerous protein factors produced by osteocytes, including sclerostin, are likely to play roles in modulating pericellular mineralization *(Atkins GJ & Findley DM, 2012)*. The full spectrum of the mode of action of sclerostin on bone is still being unraveled. It was recently published *(Kogawa M et al, 2013)* that sclerostin can target osteocytes to stimulate their resorptive activity. This suggests that osteocyte production of sclerostin may play intrinsic roles in bone remodeling and possibly calcium homeostasis. More studies are underway to increase the knowledge of these fascinating osteocytes.

1.4.3 Osteoclasts

Osteoclasts are cells responsible for bone resorption. They are characterized by their large size (20-100 µm in diameter) and presence of multiple nuclei. Mature osteoclasts are usually found in contact with calcified bone surface, within a lacuna that resulted from its own catabolic activity: Howship's lacuna. Their phenotypic features include the expression of tartrate-resistant acid phosphatase and of a calcitonine receptor. Active osteoclasts also have the capacity to form the characteristic osteoclastic ruffled membrane: i.e the so-called brush border. This complex structure resulting from extensive infoldings of the cell membrane adjacent to the bone surface, is the resorptive organelle of the osteoclast *(Holtrop ME, 1991)*.
The ruffled border is surrounded by a ring of contractile proteins (integrins) that attach the cell to the bone surface, forming an isolated microenvironment (sealing zone) beneath the osteoclast. Lysosomal enzymes, cathepsine K, metalloproteinases and ions secreted through the ruffled border allow degradation of the inorganic and organic bone matrix **(Figure 12)**.

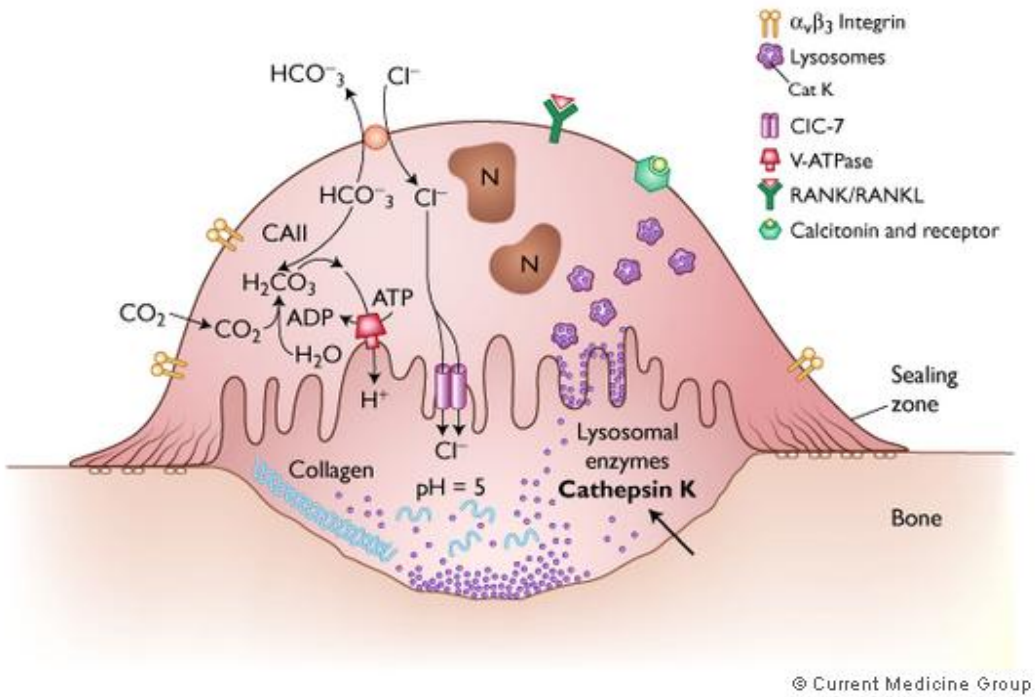


Figure 12: Mechanism of osteoclastic bone resorption (see text for details)
CO_2 : carbon dioxide; H_2CO_3: carbonic acid; H^+: proton; HCO_3^- : bicarbonate ion; CAII : Carbonic anhydrase 2; N : nucleus (Reproduced from IBMS bonekey; Rodan SB et al, 2008)

The bone degradation process **(Figure 12)** is initiated by hydration of carbon dioxide to carbonic acid, which then dissociates into protons and bicarbonate ions. The protons are transmitted into the isolated microenvironment at the bone-matrix interface via an electrogenic H^+ ATPase *(Blair HC et al, 1989; Mattsson JP et al, 1994)*. In an energy-independent manner, HCO_3^- is exchanged for Cl^- at the non-resorptive plasma membrane of the osteoclast *(Teti A et al, 1989)*. Those chloride ions entering the osteoclast pass into the resorptive microenvironment through an ion channel electrogenically coupled to H^+ ATPase *(Schlesinger PH et al, 1997)*. Thus, the osteoclast secretes hydrochloric acid into the space between itself and bone, thereby diminishing the pH of this milieu to approximately 4.5 to 5. This highly acidic microenvironment leads to mobilization of bone mineral, which exposes the organic matrix of bone:

type I collagen and noncollagenous proteins. The demineralized organic matrix is degraded by cathepsins transported to the bone–opposed plasma membrane and secreted into the isolated microenvironment.

Unlike other bone cells, osteoclasts have hematogenous origins *(Coccia PF, 1980)*, being derived from pluripotent cells in the bone marrow that also give rise to monocytes and macrophages *(Udagawa N et al, 1990)*. This explains why osteopetrotic mice could be curred after parabiosis with normal littermates *(Walker DG, 1972; Walker DG, 1973)* and administration of normal spleen cells *(Walker DG, 1975)*. A cure of human infantile malignant osteopetrosis by bone marrow transplantation was then described *(Coccia PF et al, 1980)*. These findings initiated a number of laboratories to delineate the mechanisms by which osteoclasts are recruited and resorb bone. The RANK-RANKL-OPG (Receptor Activator of Nuclear Factor κB and the ligand, osteoprotegerin) pathway appears to govern the proliferation and differentiation of the precursors into fully mature and active osteoclasts.

1.5 Bone modeling

Bone modeling, which occurs mainly in the growing skeleton and during fracture repair, is the process by which bone formation occurs, without prior bone resorption. Modeling may also occur in the adult skeleton on the outer surfaces of bone, leading to an expansion of the periosteal surface and an increase of the bone's cross-sectional area.

1.6 Bone remodeling cell cycle

In vertebrates bone remodeling is a physiological function to renew bone tissue, with a quite complex regulation *(Karsenty G & Oury F, 2010).* Bone remodeling is the continual destruction and formation of bone, a proces that occurs throughout live. In bone remodeling, bone formation and bone resorption are coupled, in that bone formation is preceded by bone resorption. The primary functions of bone remodeling in the adult skeleton are metabolic, i.e. to assist in the maintenance of calcium homeostasis, and structural, i.e. to preserve skeletal integrity by allowing bone to respond to altered mechanical loading and to repair accumulated damage. It has been estimated that 5-10% of the bone is renewed by remodeling each year. Remodeling activity is 5-10 times higher in trabecular than in cortical bone.
The basic sequence of events at any given remodeling site includes activation, resorption and formation (ARF). The phase in between resorption and formation is termed the reversal phase and the phase following the completion of the cycle the resting phase **(Figure 3)**.

This biologic process is the same in trabecular and cortical bone, though in trabecular bone it occurs on surface, wheras in cortical bone it occurs via tunneling osteoclasts, called cutting cones. The forward edge of the cutting cone consists of bone resorbing osteoclasts, followed by bone forming osteoblasts **(Figure 4)**.

1.6.1 Cellular communications

In normal circumstances the bone remodeling cycle is tightly coupled, resulting in equal amount of bone resorbed and formed. The duration of each phase varies, but generally in adult humans for any remodeling sequence, the resorptive phase lasts approximately 5-10 days, reversal 30-35 days and formation 50-100 days. During this time, a resoption pit of approximately 60-100 μm in depth has been excavated and completely refilled with new bone **(Figure 13)**.

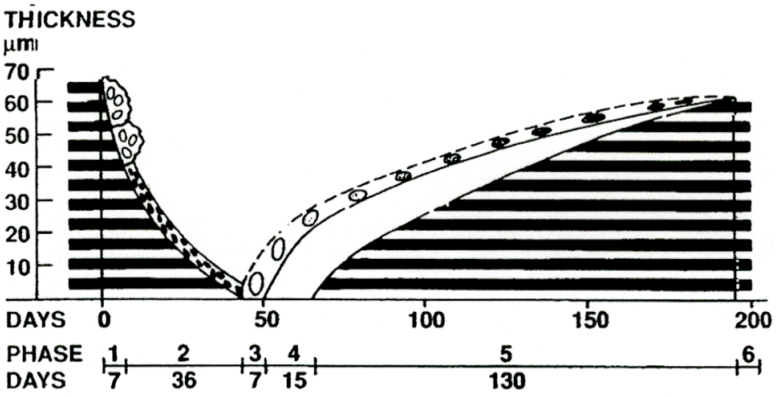


Figure 13: Normal timing of cancellous bone remodeling, including five phases : 1) osteoclastic resorption, 2) mononuclear cell resorption, 3) Preosteoblastic migration and differentiation into osteoblasts, 4) osteoblastic matrix (osteoied) formation and 5) mineralization (Reproduced from Bouxsein ML 2006, Advanced Osteoporosis Course; IOF)

1.6.2 Biochemical assessment of bone remodeling.

Bone remodeling is the result of two opposite activities, the production of new bone matrix by osteoblasts and the destruction of old bone by osteoclasts. The rates of bone production and destruction can be evaluated by either measuring osteblastic or osteclastic enzyme activities or by assaying bone matrix components released in the bloodstream and excreted in the urine. These components have been separated into markers of bone formation and of resorption. It should be kept in mind that in disease states where both events remain coupled and change in the same direction, e.g. as in osteoporosis, any marker will reflect the overall rate of bone turnover. Current bone markers cannot discriminate between turnover changes in a specific skeletal bone envelope, i.e. trabecular vs cortical, but reflect whole body net changes. Specific biochemical markers for bone remodeling have been identified *(Garnero P & Delmas JD, 2003)*. The most sensitive markers for bone formation are serum osteocalcin (OC), bone specific alkaline phosphatase (BsAP) and procollagen type I N-terminal propeptide (PINP). For the evaluation of bone resorption, immunological assays of pyridinium crosslinks of collagen have superseded total pyridinoline assays by high performance liquid chromatography (HPLC). Immunological assays are now available for pyridinoline and desoxypyridinoline (DPD) in urine and for C-terminal and N-terminal type I collagen peptides (CTX and NTX, respectively) in serum and urine. New immunoassays for serum tartrate resistant acid phosphatase (TRACP) have been developed, including the isoenzyme TRACP 5b, which is predominantly expressed by osteoclasts *(Oddie GW et al, 2000; Yam LT & Jancklia AJ, 2003)*.

1.6.2.1 Analytical and pre-analytical variability of bone markers

The analytical variability, as assessed by the interassay and intra-assay coefficients of variation, depends on the considered bone turnover marker (BTM) and the measurement method.
The pre-analytical variability has a strong effect on the BTM levels. It comprises a large number of controllable and less-controllable factors **(Table 1).** Collection of serum should be performed in standardized conditions, perferably in the fasting state in the morning. For the urine collections, the choice between a spot (usually the second morning void) and a 24-hour collection is a trade-off between the biological interest and practical reliability. The 24-hour collection reflects the overall bone metabolism, whereas the spot collection may be performed in a controlled way.
Circadian rhythm has a strong impact on the variability of BMT, especially bone resorption markers that peak in the second half of the night and have their nadir in the the afternoon (Bollen AM et al, 1995; Qvist P et al, 2002;). The amplitude is higher for CTX than for other BTM. Food intake has a strong effect on bone resoption markers. The postprandial decrease in the serum CTX is most probably mediated by glucagon-like peptide 2, the synthesis of which is stimulated by food intake (Henriksen DB et al, 2003).

Interpretation of BTM levels request the understanding of the impact of both clinical conditions and medications. These are also summarized in **Table 1**. The significant influence on bone metabolism of vitamin D, calcium status (Chapuy MC, 1996; Theiler R, 1999) and the history of recent fractures deserves special attention. The BTM levels are increased mainly for 4 months after fracture, reflecting fracture healing, and then decrease for up to 1 year.

Table 1: Determinants of pre-analytical variability of bone turnover

Controllable determinants
Circadian variation
Menstrual variation
Seasonal variation
Fasting and food intake (serum β-CTX-I)
Exercise and physical activity
Determinants that cannot be (easily) controlled :
Age
Sex
Menopausal status
Vitamin D deficit and secondary hyperparathyroidism
Diseases characterised by an acceleration of bone turnover
Primary hyperparathyroidism
Thyrotoxicosis
Acromegaly
Paget's disease
Bone metastasis
Diseases characterised by a dissociation of bone turnover
Cushing 's disease
Multiple myeloma
Diseases characterised by a low bone turnover
Hypothyroidism
Hypoparathyroidism
Hypopituitarism
Growth hormone deficit
Renal impairment
Recent fracture
Chronic diseases associated with limited mobility
Stroke / hemiplegia
Dementia
Alzheimer's disease
Medications
Oral corticosteroids
Inhaled corticosteroids
Aromatase inhibitors
Gonadotropic releasing hormone agonists
Anti-epileptic drugs
Thiazolidinediones
Heparin
Vitamin K antagonists

(Reproduced form the IOF Advanced Osteoporosis course, Lyon 2006)

1.6.2.2 Biochemical markers of bone turnover

1.6.2.2.1 Osteocalcin (OC)

Osteocalcin (OC) is a small protein synthesized exclusively by osteoblasts (and odontoblasts). It is deposited into the bone matrix to form the major non-collagenic part and can be released in part into the circulation. The flux of osteocalcin towards the serum also results from matrix resorption, thus OC is not a pure osteoblast function marker. Osteocalcin has a circadian rhythm and is higher in the early morning *(Gundberg CA et al, 1985)*. It is excreted by glomerular filtration, and its concentration is increased when glomerular filtration decreases *(Delmas PD et ak, 1983)*.
Osteocalcin can be measured by several immunoassays, but its measurement is complicated by the presence in variable amount of several osteocalcin fragments *(Diaz Diego EM et al, 1994)* resulting from its degradation in the serum. This preanalytical problem caused by a lack of conservation of serum osteocalcin can in part be solved by using an immunoassay, which recognizes a large specific N-terminal fragment *(Blumsohn A et al, 1995)*.
Differential analysis of the carboxylated or undercarboxylated form are not performed for routine clinical use.

1.6.2.2.2 Bone specific alkaline phosphatase (BsAP)

There are several isoforms of alkaline phosphatases, originating from many tissues, mainly liver and bone, with bone contributing for 40-50% of SAP in normal adults. The bone tissue enzyme can be separated from the other forms by chemical separative methods such as lectin precipitation or electrophoresis *(Van Hoof VO, 1990)*. Automation of the specific immunoassays for BsAP has improved the analytical reproducibility. Unfortunately, there is a significant cross reactivity (± 15 %) with the liver isoform *(Broyles DL, 1998)*, which can be clinically relevant when the patient suffers from liver disease. The half-life of BsAP is 1-2 days, making it less sensitive to circadian variation than other markers with a shorter half-life. The long-term intra-individual variability of BsAP is 10%.

1.6.2.2.3 Procollagen I extension peptides

Type I collagen is synthesized as a precursor, flanked at its C- and N-termini with extension peptides which are cleaved when the collagen is deposited to form the bone matrix. The catabolism of both extension peptides, procollagen 1 C terminal extension peptide (P1CP) and procollagen 1 N terminal extension peptide (P1NP), is under hormonal control and their concentration is not dependent of renal function. Because bone is remodelled faster than other connective tissues, its contribution to serum extension peptides is dominant, at least in the absence of any connective tissue disease.
Both peptides can be measured by immunoassay *(Melkko J, 1990; Melkko J, 1996)*, and have been shown to follow the expected variations of bone turnover in different physiological and pathological conditions. Circulating P1NP is rapidly degraded at 37°; recognition of the monomer varies between assays *(Brandt J, 1999)*. They follow a circadian rhythm and are higher in early morning.

1.6.2.2.4 Pyridinoline (PYD) and deoxypyridinoline crosslinks (DPD)

Aldehyde crosslinks between lysine or hydroxylysine residues are formed between collagen molecules and they stabilize the connective tissue. They are released into the circulation, and excreted into the urine, when collagen is catabolized. They reflect only collagen degradation. Deoxypyridinoline is found only in skeletal tissue but both crosslinks mainly originate from bone resorption.
When bone metabolism is normal, 50% of the crosslinks are free, and 50% bound to peptides *(Uebelhart D et al, 1991)*. The measurement of the total amount of crosslinks is most representative of true bone resorption. In high turnover bone diseases treated with anti-resorptive drugs, there was only a minimal decrease of the free crosslinks, while the peptidic forms decreased dramatically, with total crosslinks in-between *(Garnero P et al, 1995)*. Today most studies are based on immunoassay measurements, mainly of the peptidic forms. These forms follow a circadian rhythm and are higher in early morning.

1.6.2.2.5 Telopeptides of type I collagen

These peptides arise from the non-helical region of type 1 collagen where the crosslinks attach. The measured molecules are all trimeric carboxyterminal telopeptides, which are measured in serum by radioimmunoassays *(Ristelli J et al, 1991)*: i.e. type I collagen cross-linked telopeptide (ICTP) or a synthetic peptide sequence containing the crosslink site, which can be measured in serum or urine (CTX) *(Bonde M et al, 1997)*.

There are 4 isomers of CTX, according to the isomerization of the aspartate (native α- and transformed β-CTX) and to its racemisation (L or D). Both racemisation and isomerization increase with tissue age and measurement of the different forms thus gives an insight into the mean age of bone tissue with higher α/β ratios if bone turnover is increased.
Practically there are competive immunoassays, which measure the two isomers in the urine, and β-CTX can be measured in the serum with a sandwich immunoassay. Serum and urine CTX values are highly correlated.
Another assay called NTX recognizes an epitope of the N-terminal telopeptide of the α-2 chain of collagen I *(Hanson DA et al, 1992)*.
NTX and CTX telopeptide levels in serum and urine follow a circadian rhythm and show higher values in early morning.

1.6.2.2.6 Acid Phosphatases

Osteoclasts produce an acid phosphatase isoenzyme which is not inhibited by tartrate (Type 5 TRAP). Total TRAP, measured by chemical methods, has long been considered as a marker of bone resorption. However, total TRAP is influenced by enzymes originating from the erythrocytes and platelets, and its measurement can be hampered by circulating inhibitors.
At present 5 TRAP can be measured in serum by immunoassays using an immunometric format with a precision of 5% (mass measurement) or 15% (enzym activity of the captured protein).
A kinetic assay to measure specifically Type 5b TRAP, a desialylated isoenzyme present only in osteoclasts and alveolar macrophages, has also been described, with a coefficient of variation (CV) of 5-10%.
Increased type 5 TRAP levels have been described in diseases characterized by increased bone resorption, such as primary or secondary hyperparathyroidism, Paget's bone disease or metastatic bone disease.
There are few studies on TRAP 5b in osteoporosis.

1.6.2.3 Use of bone turnover markers

Bone turnover markers (BTM) improve our understanding of the relationship between bone turnover, BMD, bone fragility and the effect of an anti-osteoporotic treatment : its metabolic effect and the anti-fracture efficacy. However, practical guidelines for the use of BTM in the clinical management of osteoporosis are still lacking.

1.6.2.3.1 Biochemical markers and rate of bone loss

Several cross-sectional studies indicate that bone turnover (BT) increases rapidly after the menopause *(Garnero P et al, 1996)*. Bone mineral density (BMD) measured at various skeletal sites correlate negatively with bone turnover in postmenopausal (PMP) women, a relation which becomes stronger with the years after menopause *(Garnero P et al, 1996)*. These data suggest that a sustained increase of BT in PMP women induces faster bone loss and therefore an increased risk for osteoporosis.
Longitudinal studies, necessary to avoid potential confounding factors, suffer however from methodological issues leading to conflicting results *(Stepan JJ, 2000)*. When bone loss is assessed by measurement of BMD over 2-4 years, the amount of bone loss is of the same magnitude as the precision

error of repeated measurements in a single individual i.e. 3-4%. When reducing the precision error of the rate of bone loss by multiple repeated BMD measurements over e.g. 2 years, the correlation coefficient with bone markers tends to improve from about -0.3 to -0.8 *(Johansen JS et al, 1988)*.

1.6.2.3.2 Markers of bone turnover and fracture risk

Prospective studies have looked at the relationships between the measurement of biochemical markers of bone formation and bone resorption and the subsequent riks of fractures *(Garnero P, 2000a)*. The studies investigating the relationships between bone formation markers and fracture risk have yielded conflicting results.
More consistant data have been obtained on the relationship between increased levels of bone resorption markers and fracture risk.
Four prospective studies (Rotterdam, EPIDOS, OFELY and Hawai osteoporosis study (HOS)) have demonstrated that bone resorption, assessed by urinary or serum CTX or by urinay desoxypyridinoline levels above the premenopausal range were consistently associated with about a two-fold higher risk of hip, vertebral and nonhip nonvertebral fractures over follow-up periods ranging from 1,8 to 5 years *(Garnero P et al, 1996a; van Daele PLA et al, 1996; Garnero P, 2000a; Garnero P et al, 2000b; Chapurlat RD et al, 2000)*.
Because serum CTX levels are markedly affected by food intake *(Qvist P et al, 2002; Bjarnason NH et al, 2002)*, an effect which is likely to be mediated by gastrointestinal hormones *(Henriksen DB, 2003)*, it should be measured on fasting morning samples to reduce the variability of the measurement *(Delmas PD et al, 2000a)*.
This technical limitation probably explains discordant results on fracture prediction of CTX, when measured on nonfasting samples (i.e. the EPIDOS *(Chapurlat RD et al, 2000)* and SOF *(Bauer DC et al, 1999)* studies).
Increased bone resorption is associated with increased fracture risk only for values which exceeds the upper limit of the premenopausal range. This suggest that increased bone resorption above the normal physiological threshold, could lead to increased skeletal fragility through two independent mechanisms. First, a prolonged increase of BT will lead after several years to a lower BMD, which is a major determinant of reduced bone strength. Secondly, considerable increase of BT may induce architectural deterioration of bone tissue such as perforation of trabeculae, a major component of bone strength.

Guidelines (Bergmann P et al, 2009) recommend the combined use of 2 bone turnover markers (BTMx): one for the evaluation of bone formation and one for the evaluation of bone resorption. Due to the quality of the assays and the degree of standardization the reference BTMs are serum procollagen type I N propeptide (s-PINP) for bone formation and serum C-terminal cross-linking telopeptide of type I collagen (s-CTX) for bone resorption.
A recent systematic review and meta-analysis (Johansson H et al, 2014) of longitudinal prospective studies examined the performance characteristics of s-PINP and s-CTX in fracture prediction in untreated individuals. Ten studies were indentified and 6 studies were included in the analysis. There was a modest but significant association between BTMs and risk of future fractures. The hazard ratio per SD increase in s-PINP (gradient of risk (GR)) was 1.23 (95% CI 1.09 - 1.39) for men and women combined unadjusted for bone mineral density. There was also a significant association between s-CTX and risk of fracture, GR= 1.18 (95% CI 1.05 - 1.34) unadjusted for bone mineral density.

1.6.2.3.3 Posttranslational modifications of bone matrix proteins

Type I collagen molecules undergo enzymatic and nonenzymatic posttranslational modifications that may be of clinical relevance for the investigation of metabolic bone diseases.
Among the enzymatic modifications, biochemical studies on humans bone specimens have shown that an over-hydroxylation of lysine residues, an over-glycosylation of hydroxlysine and a reduction in nonreducible crosslinks can be associated with reduced bone strength *(Bätge B et al, 1992; Kowitz J et al, 1992; Bailey AJ et al, 1992; Oxlund H et al, 1996)*. The ratio of pyrodinoline (PYD)/desoxypyrodinoline (DPD) was significantly associated with the compressive biomechanical properties of the vertebrae independently of BMD *(Banse X et al, 2002)*.
Non-enzymatic advanced glycation, assessed by pentosidine concentration in bone increases with age and is associated with decreased bone strength *(Wang X, 2002)*. Another non-enzymatic posttranslational

modification is the β-isomerization of the aspartate residu of CTX (see above). The degree of bone type I collagen isomerization can be detected in vivo by the differential measurement of native (α) and isomerized β-CTX fragments in the urine *(Garnero P et al, 1998a)*. In a prospective study the ratio between native and β-isomerized CTX in urine was significantly associated with increased fracture risk independently of both the hip BMD and bone turnover rate as measured by serum BsAP *(Garnero P et al, 2002)*.
Osteocalcin contains three residues of γ-carboxyglutamic acid (GLA), a vitamin K-dependent amino acid. Vitamin K2 treatment in postmenopausal women with osteoporosis resulted in decreased serum levels of undercarboxylated ostecalcin (unOC), in an increased spine BMD and in a reduced risk of fragility fractures *(Shiraki M et al, 2000)*.
In prospective studies levels of unOC above the premenopausal range was associated with a two to three fold increase in the risk of hip fractures; even after adjustment for hip BMD, although total OC was not predictive *(Szulc P et al, 1993; Szulc P et al, 1996; Vergnaud P et al, 1997)*.

1.6.2.3.4 Combined assessment of fracture risk

Several prospective studies in postmenopausal women demonstrate that addition of bone turnover levels improve fracture prediction models based on clinical risk factors. Combination of a single clinical risk factor, i.e. history of fragility fracture after 45 years, with a low hip BMD and high levels of bone resorption as assessed by urinary CTX improved the predictive value of the relative risk of fracture from 1.8 -2.8 to 5.8 *(Garnero P et al, 2000b)*.
In a nested case control study, the combination of urinary CTX with either hip BMD or heel broadband ultrasound attenuation (BUA) increased the specificity of the predictive value by 10% with a sensitivity similar to BMD or BUA alone *(Garnero P et al, 1998b)*.
If DXA or ultrasound is not available, the combination of a high bone resorption marker and a positive history of any type of frature gave a predictive value similar to that obtained by BMD or BUA alone (*Garnero P et al, 1998b)*. Bone markers may be used in the assessment of fracture risk in selected cases in which BMD and clinical risk factors are not enough to take a treatment decision.

1.6.2.3.5 Bone turnover markers for initiating and monitoring treatment of osteoporosis

BTM do not appear to be a significant determinant in the selection of a particular anti-osteoporosis drug. The anti-fracture efficacy of anti-osteoporosis agents appears to be
largely independent of baseline bone turnover (Reginster JY, 2008).

Monitoring individual patients' treatment is essential in every patient with a chronic disease. More specifically in bone disease, e.g. in osteoporosis, failure to respond to treatment can be due to compliance problems, poor intestinal absorption, other various factors contributing to bone loss or unidentified factors. Follow-up of BMD by bone densitometry as a single measure is found not adequate nor validated for monitoring leading to treatment change decisions. The main reason herefore is that the time to reach clinical relevant significant changes is in the range of 1 to 2 years. Secondly, in reanalyses of placebo controlled intervention studies with antiresorptive osteoporosis drugs BMD changes are not predictive for changes in fracture risk. They merely explain only a minor part of the treatment's efficay on fracture risk *(Cummings SR et al, 2002)*.

This observation opens an opportunity for bone turnover markers to come into the field *(Bergmann P et al, 2009)*. Bone resorption inhibiting therapy is associated with a prompt decrease of bone resorption markers as early as 1 month with a plateau reached within 3 to 6 months after treatment initiation. In several placebo-controlled studies it has been demonstrated that the magnitude of decrease of the bone turnover markers under antiresorptive therapy is not only strongly correlated to the increase in BMD after 2 to 3 years *(Delmas PD, 2000b)* but also with reduction in subsequent vertebral fracture risk *(Eastell R et al, 2003)* or subsequent vertebral, hip and nonspine fracture *(Bauer DC, 2003)*.
The relationship between the reduction in vertebral fracture risk and changes from baseline bone resorption markers during 3 months was not linear. There was a level of bone resorption reduction below which no future fracture benefit was observed. In clinical practice, recommended values of bone marker

changes with treatment should still be developped in prospective studies with incident fractures as an endpoint. An issue in this respect is the standardization of tests, control of its preanalytical variance and reduction of analytical reproducibility.

The early decrease in BTM levels (6-12 months) is associated with the long-term antifracture efficacy (2-3 year) of an antiresorptive agent (Bauer DC et al, 2004; Reginster JY et al, 2004; Eastell R et al, 2007). The early increase of BTM in bone formation-stimulating treatment, e.g. teriparatide, is correlated positively with the subsequent increase in BMD, especially with the increase in trabecular volumetric BMD (Chen P et al, 2005).
Withdrawal of an osteoporosis treatments is rapidely followed by a return of BTM values to baseline values in case of hormonal replacement therapy or shortterm bisphosphonate treatment (Sornay-Rendu E et al, 2003; Ravn P et al, 1998). This return to baseline values occur within a more protracted time period in case of longterm and high affinitiy bisphosphonates as e.g. alendronate, zoledronate (Black DM et al, 2006).
Low compliance is a serious problem during anti-osteoporotic treatment, leading to an increased risk of fracture (Caro JJ et al, 2004). The better the compliance of risedronate treatment, the greater the average decrease in bone turnover (Eastell R et al, 2003b). Receiving positive feedback corresponding to a > 30% decrease in urinary NTX, resulted in lower risk of treatment discontinuation (Delmas PD et al, 2007).

Currently available pharmacological treatments for osteoporosis promptly and significantly impact on the rate of bone turnover. Short-term changes in BTM reflecting bone resorption (for raloxifene, bisphosphonates) or bone formation (for raloxifene, teriparatide, 1-84 parathryoid hormone) have been consistently shown to predict future BMD changes (for bone forming agents) or long-term fracture reduction (for anti-resorptive agents), strongly suggesting that BTM could play an significant role in monitoring of anti-osteoporosis therapy (Reginster JY, 2008). Some (Bergmann P et al, 2009), but not all guidelines or expert opinions support this view. Further research should be stimulated to assess the relevance of these findings to the care of individual patients, i.e. how to define the response to a specific treatment based on the short-term changes observed in BTM.

1.6.2.3.6 Bone turnover markers in men

In boys, the growth spurt starts later and lasts longer than in girls. Therefore, young men enter the phase of consolidation, i.e. the formation of peak BMD after growth arrest, later than women. Men have wider bones even after adjustment for body size. At the age of 20-25 year, BTM levels in men are higher than in women because men have more active bone turnover in longer and wider bones. Then, BMUs decrease and attain their lowest levels between 50 to 60 years of age (Khosla S et al, 1998; Fatayerji D et al, 1999; Szulc P et al, 2001). Bone resorption increases progressively after the age of 60. Men with high bone turnover have lower BMD; thus age related bone loss in men may result at least in part from increased bone resorption. Older men with high bone turnover have a faster subsequent bone loss. However, recent prospective large cohort studies showed that BTM do not predict osteoporotic fractures in older men (Szulc P et al, 2008; Bauer DC et al, 2009).

1.6.3 Balance and frequency of the bone remodeling cycle

During a bone remodeling cycle in pathologic conditions, bone resorption often exceeds bone formation. The metabolic activity in each remodeling site thus results in a net deficit in bone mass or in a negative bone balance, e.g. in menopausal women, in the acute phase of male hypogonadism or under high dose corticosteroid therapy, hyperthyroidism, etc ... .
Administration of antiresorptive or anabolic agents may temporarily alter the coupling between bone formation and resorption. Treatment with a bisphosphonate first decreases bone resorption and subsequently bone formation.
Because there is a time interval between the onset of the inhibition of the resorption process and the secondary delayed decrease of bone formation, bone formation at current sites will proceed, leading to a net gain in bone mass. This phenomenon is termed the “filling of the remodeling space”. Further increase of bone mineral density following reduction in bone turnover is likely due to increased secondary mineralization of existing bone, rather than formation and mineralization of new bone matrix.

Bone remodeling serves a metabolic role in liberating calcium from the bone matrix into circulation. Although there are other target organs to accomplish mineral homestasis: i.e. the intestine and kidney, 99% of the body calcium is stored in the skeleton. Skeletal remodeling thus plays a key role in maintaining ionized blood calcium levels.

A second factor thought to govern bone remodeling is the need to maintain skeletal integrity by providing a mechanism for bone to respond to increased or decreased mechanical loading, and to repair accumulated microdamage. The internal architecture of bone, i.e. the trabecular struts, is aligned along the primary loading directions. This ability of bone to undergo structural reorganization according to mechanical stimuli was formulated in 1892 by Julius Wolff *(Wolff J, 1986)*, and became known as "Wolff's Law". The unraveling of the precise mechanisms underlying the bone's remarkable ability to self-regulate its external and internal geometries has only been started in the last 2 decades. Harald Frost *(Frost H, 1987)* extended his theories of bone adaptation of mechanical force to include the concept of the "Bone Mechanostat" stating that specific amounts of bone strain are required to induce phases of bone modeling, bone remodeling and bone loss **(Figure 14)**. He proposed that bone structure is regulated by local mechanical effects that these effects are adjusted by system hormones, just as you would adjust a thermostat. The feedback control system in a steady state, where bone structure is maintained, such that ordinary mechanical strains do not exceed a minimum effective strain (MES). If the local strains within the bone surpass the MES, bone will undergo modeling and change its structure to reduce the local strains to below the MES. Hormones or biochemical agents, that could sensitize bone cells so that the MES threshold is lowered to normal mechanical usage, would increase bone mass and bone strength significantly.

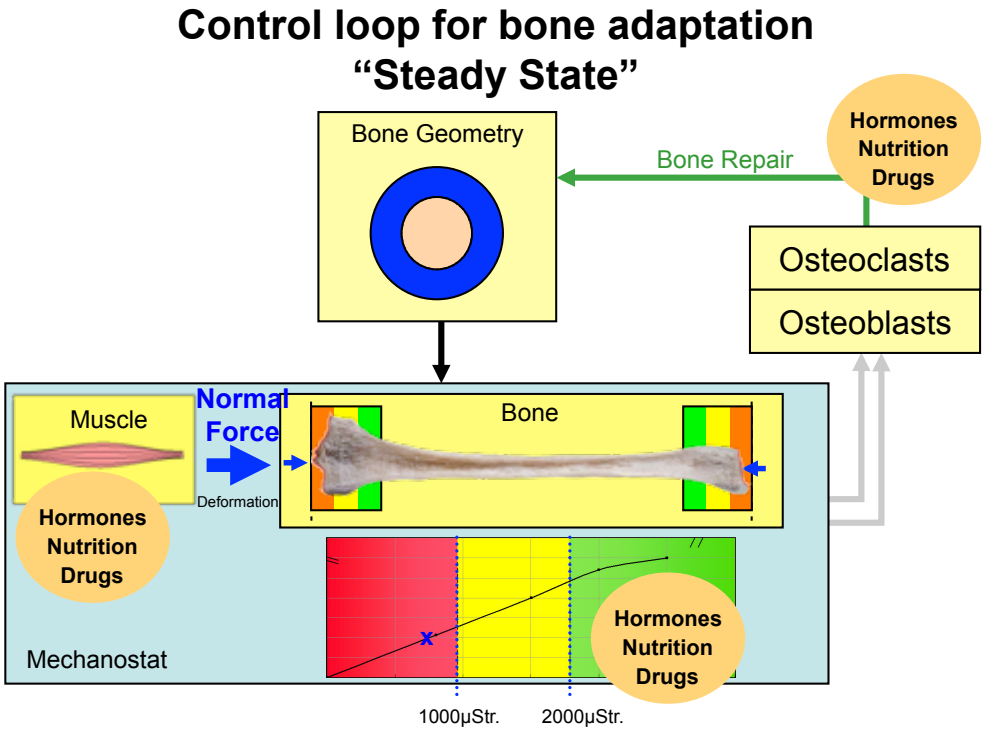


Figure 14: The bone mechanostat. In a steady state of bone remodeling the applied strain in the bone by forces is usually between 1000 and 2000 µstrain. Lower strains by disuse leads to bone resorption (mainly at the endocortical envelop); exercise inducing higher strains result in bone gain (modeling mainly at the periosteal envelop). Hormones, nutrition and drugs may reset the thresholds: by for at a see text for description (Reproduced from Frost H, 1987)

The hypothesis of a mechanically-dedicated message traffic and feedback in the mechanostat must be supported by 4 aspects. Firstly, biological mechanisms that determine skeletal health and disease need effector cells and non-mechanical agents in order to work, as a care need fuel, engines and wheels in order

to move. Secondly mechanical factors that guide those mechanisms in time and space are needed, as drivers, steering, brakes and accelerators do for cars. Thirdly, neuromotor physiology and anatomy are required to dominate control of those biological mechanisms. Lastly, non-mechanical factors can help or hinder, but cannot replace or duplicate the mechanical control.

The perception by the osteocytes of loading induced fluid shear stresses and their biochemical response are thought to be the biological basis for the mechanostat theory. Most probably these responses are under genetic and environmental control.
Another mechanical stimulus that appears to regulate bone remodeling is the presence of microcracks in the bone matrix *(Mori S & Burr DB, 1993)*. It has been demonstrated that new remodeling events are more likely to occur at the site of a microcrack (targeted remodeling) than anywhere else. The extent to which this directed remodeling is impaired with age, and consequently its contribution to skeletal integrity remains to be determined. It is speculated that the longterm reduction of bone turnover by antiresorptive therapies might inhibit repair of microdamage and eventually enhance skeletal fragility. The optimal level of bone remodeling and the safe levels of remodeling suppression have yet to be determined.
Another biomechanical concept is the muscle-bone relationship. Muscle action is, via amplification of the applied force by leverarms in the locomotor system, responsible of the major part of the force applied to bone. Muscle force plays an important role in the adaptation of bone. Due its adaptive capacity bone mass is directly tied to lean muscle mass and muscle force. A lot of controversy on this topic araised because it was widely misunderstood, that the concept ignored the influences of hormonal and metabolic factors in bone physiology. Howver, it did not ignore these well-known influences, but it provides a means to determine whether the primary abnormality in a given osteopenia is related to chiefly muscle pathology or bone pathology.

1.6.4 Regulatory molecule pathways

The molecular basis of the intracellular communciation in bone tissues became more and more unraveled within the last two decades. The main pathways are the RANK/RANKL/OPG pathway and the osteblastogenesis control feedback loop including sclerostin. However, other minor pathways are involved and still need more investigation.

1.6.4.1 RANK/RANKL/OPG pathway

The basis of the coupling between bone formation and resorption is comprised of three main molecular communication systems between osteoblasts and osteoclasts **(Figure 15)**.

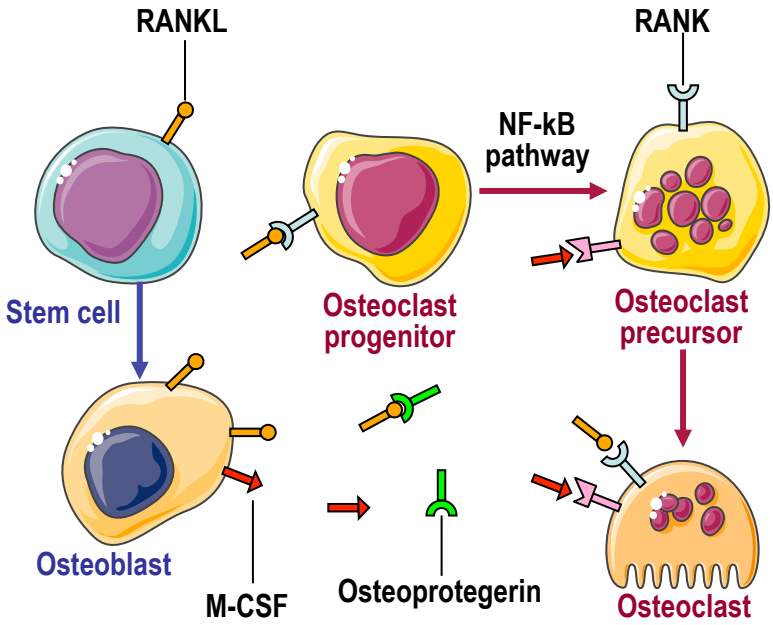


Figure 15: RANK/RANKL/OPG pathway: see text for description (Reproduced from Friedlander G, 2012)

The first one is Macrophage Colony Stimulating Factor (M-CSF), a cytokine produced by osteoblasts which binds to osteoclast precursors and promotes their recruitment.
The second system consists of the Receptor Activator of Nuclear factor κB (RANK) and its Receptor Activator of Nuclear factor κB ligand (RANKL). Osteoblasts and marrow stromal stem cells produce RANKL which can circulate and bind to RANK, located at the surface of osteoclasts and osteoclast precursors. Differentiation of osteoclasts proceeds when RANKL binds to RANK. In case of loss-of-function mutation of RANKL, the recruitment, differentiation and function of osteoclast will not occur properly. This results in a form of osteopetrosis characterized by a very low number of osteoclasts in bone.
Thirdly, and critical in the RANK/RANKL pathway is osteoprotegerin (OPG), a circulating peptide synthesized by stromal cells and osteoblasts. OPG acts as a decoy receptor of RANKL and inhibits osteoclastogenesis and bone resorption. OPG is therefore a fine tuner of the osteoblast-osteoclast cross-talk.

Although playing a role as local paracrine factors in the bone, concentration of RANKL and OPG in the plasma of patients can be measured and are potentially interesting markers of bone remodelling.
Whereas in osteoclasts, activation of NF-κB by RANKL/RANK interaction promotes bone resorption, NF-κB signaling in osteoblasts decreases bone formation and subsequently bone mass. Inhibition of NF-κB on the contrary, acts through enhancing the expression of Fos-related antigen-1 (Fra-1), an essential transcription factor involved in bone matrix formation. So, targeting NF-κB this way, may help promoting bone formation in pathological situations such as osteoporosis and other bone diseases.

1.6.4.2 Wnt/β-catenin signaling pathway

In humans, Wnts are a family of 19 cysteine-rich secreted glycoproteins with a function in a plethora of cellular actions including embryonic and postnatal development, induction of cell polarity, maintenance of tissue homeostasis and cell growth control *(Miller JR et al, 2002)*. Wnt signal transduction is mediated through interaction with Frizzeld (Fz) receptors and interaction with additional co-receptors like low density lipoprotein receptor related protein 5 or 6 (LRP5/6) **(Figure 16)**. Best understood is the Wnt/β-catenin signalling canonical pathway of which we provide a short overview of the extracellular and

transmembrane modulators. The intracellular signaling pathway is not discussed here. The Wnt/β-catenin pathway is involved in maintaining proper mammalian bone homeostasis. Canonical Wnt/beta-catenin signalling promotes osteoblast differentiation from mesenchymal stem cells.

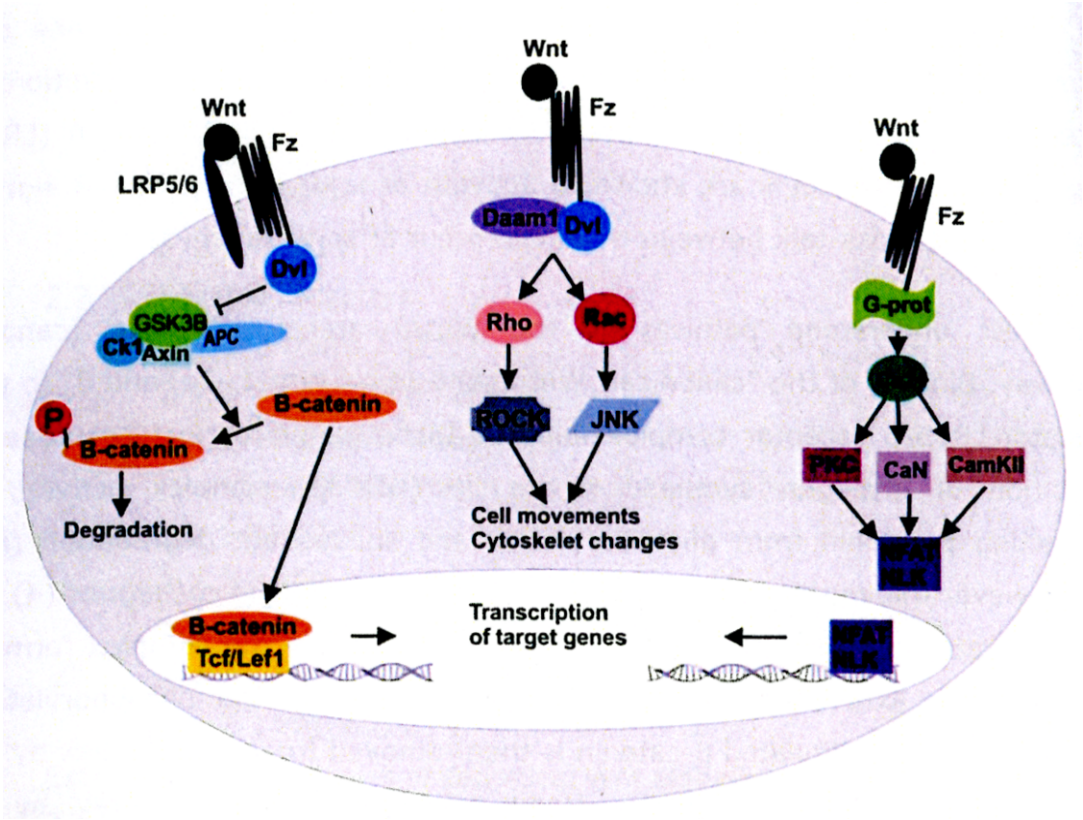


Figure 16: Overview of the three different Wnt signalling pathways.
Activation of the canonical Wnt signalling pathway (left) results in an inhibition of the protein complex formed by GSK3B, APC, CK1 and axin and consequently results in increased β-catenin levels in the cytoplasma. Activation of the non-canonical PCP Wnt pathway (middle) and calcium dependent (right) Wnt pathway by Wnt results in activation of Rho/Rac GTPases and PKC/CaN, CamKII, respectively (Reproduced from Boudin E, 2013).

1.6.4.2.1 Wnt ligands

The highly conserved int1 gene, as was discovered in mice in 1982, was in fact a previously known and characterized Drosophila gene, known as Wingless (Wg) *(Nusse R & Varmus H, 2012)*. The int/Wingless family was renamed the Wnt family, as int became Wnt, which stands for Wingless-related integration site.

Wnt ligands are secreted glycoproteins. In humans these consisting of 19 members, which can activate the three Wnt signaling pathways by binding Frizzled (FZ) and if necessary additional co-receptors. The downstream "canonical" and "non-canonical" pathway **(Figure 16)** relies on specific receptors *(van Amerongen R et al, 2008)* and/or on the available amount of the Wnt ligand *(Nalesso G et al, 2011)*.

1.6.4.2.2 Frizzled receptors

Frizzled (Fz) proteins are seven-pass membrane receptors, which can activate canonical and non-canonical Wnt signaling **(Figure 16)**. In humans the Fz family contains 10 members and it is suggested that not all Fz proteins have the same function in Wnt signalling indicating the existence of inhibitory and stimulatory Fz proteins *(Westendorf JJ et al, 2004)*.

1.6.4.2.3 Low density-lipoprotein receptor related proteins

The low density-lipoprotein related protein (LRP) family is a group of evolutionary conserved cell-surface receptors with a function in a range of cellular processes *(Nykjaer A & Willnow TE, 2002)*. Studies have shown that various LRPs are involved in the regulation of osteoblast function and consequently in the regulation of bone mass.

1.6.4.2.3.1 LRP5

Initial evidence that LRP5 is a major factor in the regulation of osteoblast proliferation and differentiation, osteocyte apoptosis and bone formation, came from positional cloning studies on monogenetic bone disorders. Loss-of-function mutations in LRP5 were shown to be causative for osteoporosis pseudoglioma (OPPG), a disease marked by a reduced bone mass and blindness *(Capoen J et al, 1993; De Paepe A et al, 1993; Gong Y et al, 2001; Korvala J et al, 2012)*. Furthermore, gain-of-function mutations were found to result in different high bone mass (HBM) phenotypes, all characterized by an increased cortical thickness of the long bones and the skull: so-called craniotubular hyperostosis *(Boyden LM et al, 2002; Little RD et al, 2002; Van Wesenbeeck L et al, 2003)*. Functional studies showed that these HBM mutations result in a decreased binding of the Wnt signalling inhibitors sclerostin and dickkopf 1 (Dkk1) *(Johnson ML et al, 2004; Balemans W et al, 2007; Bhat BM et al, 2007; Balemans W et al, 2008; Pangrazio A et al, 2011)*.
More recently it has been demonstrated that LRP5 and Wnt/β-catenin signalling are required for osteogenesis in response to mechanical loading *(Robinson JA et al, 2006; Hens JR et al, 2005; Lau KH et al, 2006; Sawakami K et al, 2006)*. This is probably most relevant in the osteocyte's capability to transmit signals of loading to cells on the bone surface *(Bonewald LF & Johnson ML, 2008)*. Recently, it was proposed that LRP5, instead of having a principle role in osteoblasts, regulated bone formation and bone mass accrual by inhibiting tryptophan hydroxylase 1 (TPH1) and serotonin synthesis in the duodenum *(Cui Y, 2011 et al; Yadav VK et al, 2010)*. For further explanation see paragraph 1.7.1.1.5.

1.6.4.2.3.2 LRP6

LRP6 is a close homologue of LRP5. Both proteins share 71% amino acid identity and are in part functionally redundant *(Holmen SL et al, 2004; Williams BO & Isogna KL, 2009; Joeng KS et al, 2011)*. In contrast to LRP5 knockout mice, global deletion of LRP6 is not viable. As shown in in-vitro and in-vivo studies in mice, LRP6 also plays a role in the regulation of bone metabolism in humans *(Mani A et al, 2007)*. Most association studies showed that common variations in the LRP6 gene are associated with higher BMD and lower fracture risk *(Riancho JA et al, 2001; van Meurs JB et al, 2006; Sims AM et al, 2008; Mencej-Bedrac S et al, 2009)*.

1.6.4.2.3.3 Other LRP

LRP 4 shares homology with LRP5/6 in its extracellular domain, but its cytoplasmic domain contains endocytosis signals, that have been suggested to mediate the internalization of proteins and subsequent degradation/recycling *(Strickland DK & Ranganathan S, 2003)*. Common variations in the LRP4 gene were shown to be associated with osteoporosis-related phenotypes, like BMD and fracture risk, in both candidate gene and genome wide association studies *(Richards JB et al, 2009; Rivadeneire F et al, 2009; Kumar J et al, 2012)*.
Also LRP1/2/8 have been described, but have been less extensively studied in contrast to LRP4/5/6. The importance of these more recently detected receptors in the Wnt signalling pathway is demonstrated by a variety of bone phenotypes.

1.6.4.3 Extracellular mediators of the canonical Wnt signalling in bone

Canonical Wnt signalling is a tightly modulated pathway. Modulation can take place intracellularly as well as extracellularly *(Piters E et al, 2008; Monroe DG et al, 2011)*. In the following section the focus is on secreted extracellular modulators. These can be divided into two groups; on the one hand, proteins that modulate the pathway by binding to LRP5/6 co-receptors and on the other hand those that modulate the binding to Wnt **(Figure 17).**

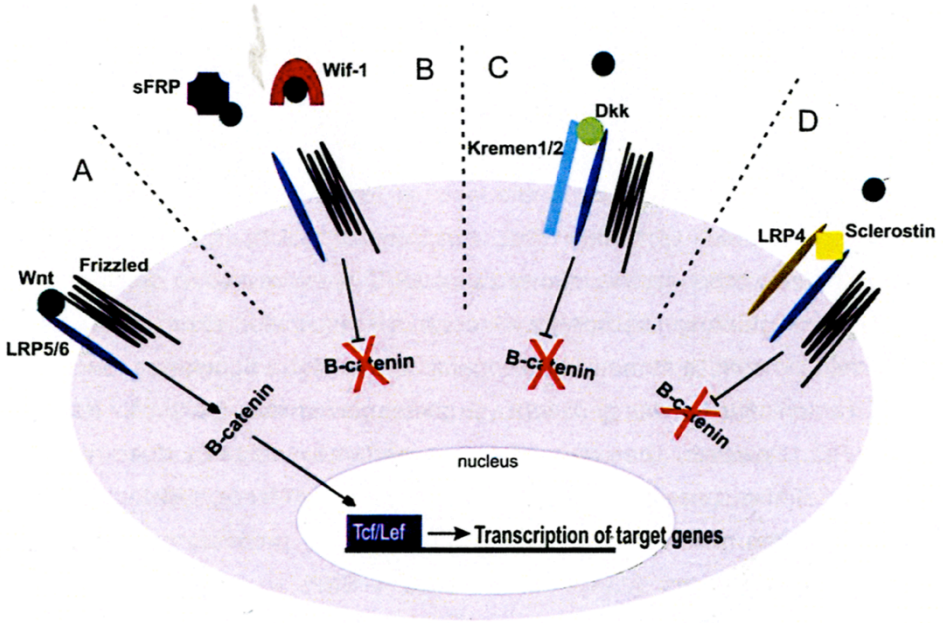


Figure 17: Extracellular modulation of the Wnt/β-catenin dependent Wnt signalling.
A: Activation of the pathway by binding of Wnt on the LRP5/6-Fz receptor complex.
B: Inhibtion of the Wnt pathway by binding directly to Wnt ligands (sFRP family and Wif-1). C+D: Inhibition of the canonical Wnt signalling by binding of Dkk or sclerostin to Kremen1/2 and LRP4, respectively (Reproduced from Boudin E, 2013).

1.6.4.3.1 Secreted frizzled related proteins

Secreted frizzled-related proteins (sFRPs) antagonize Wnt signaling (canonical and non-canonical) by binding to Wnt protein and consequently preventing Wnt/receptor activation.
Systemic overexpression of sFRP1 led to a reduced Wnt/β-catenin signaling in bones. This is associated with reduced osteoblast activity and bone formation. Therefore, mice overexpressing sFRP1 developed osteopenia. PTH administration leads to Wnt/b-catenin pathway activation in bone. sFRP1 overexpression impaired this PTH induced Wnt/b-catenin activation and blunted bone response to PTH **(Figure 18)**.

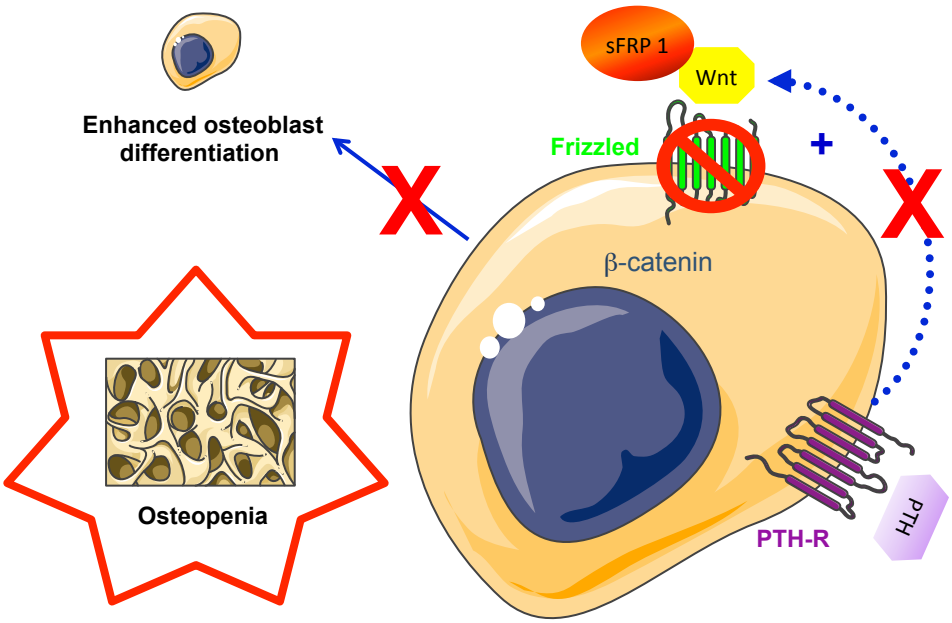


Figure 18: Regulation of canonical Wnt signalling in bone metabolism: sFRP1 inhibits bone formation and attenuates PTH bone anabolic effects (Reproduced from Friedlander G 2012).

However, the functions of sFRPs in Wnt signalling seem to be more complex *(Mii Y & Taira M, 2011)*, and more in depth research is important for further development of potential molecular targets for anabolic therapy in osteoporosis and fracture healing. In general, for several sFRPs, e.g. sFRP1 and sFRP4, clinical association studies show that common genetic variation is associated with bone mineral density at different sites, as well as with hip geometry parameters *(Sims AM et al, 2008; Ohnaka K et al, 2009; Boudin E et al, 2012)*.

1.6.4.3.2 Wnt inhibitory factor 1

Wnt inhibitory factor 1 (Wif-1) is an evolutionary conserved protein that, similar to sFRPs, binds to Wnt proteins and inhibit Wnt signalling. Based on in-vitro study data, it has been suggested that Wif-1 is part of a negative feedback loop that controls osteblast differentiation and maturation *(Cho SW et al, 2009)*. It was demonstrated that Wif1 is epigenetically silenced in human osteosarcomas, suggesting Wif1 is a tumor suppressor gen *(Kansara M et al, 2009; Enders GH, 2009)*.

1.6.4.3.3 Glypicans

A family of proteins that can bind Wnt ligands are the glypicans (Gpc), a series of glycosylphosphatodylinositol (GPI)-anchored cell-surface proteoglycans (PG). Gpcs are regulators of ligand-receptor encounters and thereby control development through several signalling pathways including Wnt signalling. Depending on the cellular context Gpcs can act as inhibitors or stimulators on different pathways *(Filmus J et al, 2008)*. Gpc1, 3 and 4 have shown to be involved in the regulation of bone metabolism, however, the precise mechanisms remain unclear. Sclersostin for example has a potential binding domain for heparan sulphate proteoglycans such as glypicans *(Veverka V et al, 2009).* In addition, Gpc4 is shown to bind to Dickkopf-1 (see below) *(Caneparo L et al, 2007)*, and Gpcs are shown to be involved in other pathways: e.g. the hedgehog pathway *(Capurro MI et al, 2009)*.

1.6.4.3.4 SOST – sclerostin

The evidence that the SOST gene, which encodes the protein sclerostin, is involved in the regulation of bone formation is delivered by the identification of disease causing mutations in patients diagnosed with sclerosteosis. This genetic condition with an autosomal recessive manner of inheritance is characterized by a progressive bone overgrowth especially at the skull, mandibule and tubular bones. Patients with sclerosteosis have large stature and syndactyly *(Beighton P et al, 1977; Brunkow ME et al, 2001; Balemans W et al, 2001; Balemans W et al, 2005; Piters E et al, 2010)*.
Van Buchem disease, which is resembling sclerosteosis, however with a milder phenotype, is caused be a 52 kb deletion downstream to SOST *(Balemans W, 2002)?*
This deletion results in a decreased transcription of SOST *(Loots GG et al, 2005; Collette NM et al, 2012)*.

Sclerostin is predominantly expressed by osteocytes and antagonizes canonical Wnt signalling by binding LRP5/6 and preventing the activation by Wnt proteins **(Figure 17)** *(Loots GG et al, 2005; Collette NM et al, 2012)*. This inhibitory effect of sclerostin on Wnt/β-catenin signalling is disrupted in the presence of the previously mentioned HBM-causing mutations of the first β-propeller of LRP5. This region is responsible for the binding with sclerostin *(Balemans W et al, 2008; Pangrazio A et al, 2011)*.
By the discovery of SOST as a disease causing gene for sclerosteosis and Van Buchem's disease, sclerostin and Wnt/β-catenin signalling became interesting targets for further research in order to identify the regulatory mechanism of bone homestasis.
SOST knockout mice have a high bone mass phenotype with an increase of bone mineral density (BMD), bone volume and bone strength due to elevated bone formation *(Li X et al, 2008; Krause C et al, 2010)*.
SOST overexpression mice on the contrary, have an impaired BMD, bone volume and strength due to decreased bone formation rates *(Winkler DG et al, 2003; Loots GG et al, 2005)*.
Sclerostin is involved in the anabolic effect of PTH, as is demonstrated by the decline of sclerostin levels following PTH infusion in healthy men *(Yu EW et al, 2011)*. The only currently available therapy to stimulate bone formation in humans, intermittent PTH administration, was shown to reduce SOST mRNA and sclerostin protein expression in rats and mice *(Keller H et al, 2005; Bellido T et al, 2005)*. This suggests that inhibition of sclerostin expression, thereby removing a negative regulator of Wnt-stimulated bone formation, may play a role in the stimulating effect of intermittent PTH on bone formation.

Several in-vitro and in-vivo studies showed that sclerostin is involved in the osteogenic response to mechanical loading since loading reduces the expression of SOST by osteocytes resulting in increased canonical Wnt signaling leading to increased bone formation *(Li X et al, 2008; Yu EW et al, 2011)*.
In addition to the involvement of SOST in monogenetic diseases, several studies showed that variation in SOST is associated with BMD and osteoporotic fractures *(Sims AM et al, 2008; Richards JB et al, 2009; Huang QY et al, 2009; Yerges, LM et al, 2009; Piters E et al, 2012)*.

1.6.4.3.5 SOST-DC1

SOST-DC1 (sclerostin domain containing factor 1), alternatively called ectodin, wise or usag-1, is a secreted protein and has a dual role in modulating Wnt signaling *(Itasaki N et al, 2003; Lintern KB et al, 2009)*. It is an interesting protein for further research regarding its function in regulating bone mass.

1.6.4.3.6 Dickkopf proteins

The Dickkopf (Dkk) family encodes for secreted glycoproteins and consists of 4 members (Dkk1-4). Dkks can inhibit the canonical Wnt signaling by forming a complex between LRP5/6 and Kremen1/2 (krm1/2) **(Figure 17)** *(Wang K et al, 2008)*.

1.6.4.3.7 Kremen

Kremen 1 and 2 (Krm1/2) are single pass trans-membrane receptors with high affinity for Dkk proteins. Krm1/2 can form a trimolecular complex with Dkk and LRP5/6 which is rapidely endocytosed resulting in

inhibition of the canonical Wnt signaling pathway **(Figure 17)** *(Mao B et al, 2002)*. It is also demonstrated that Krms can promote this pathway by binding directly to LRP6 in absence of Dkk1.

1.6.4.3.8 R-spondins

The R-spondin family of secreted Wnt agonist consist of 4 members (Rspo 1-4) *(Kim KA et al, 2006)*. They can synergize with Wnt ligands by stabilizing of cytosolic β-catenin levels, leading to an up-regulation in Wnt/β-catenin signaling *(Wei Q et al, 2007; Kazanskaya O et al, 2004; Binnerts ME et al, 2007)*. Strong evidence suggests that R-spondins are capable of binding to LRP6 and thereby disrupting the LRP6/Dkk/Krm complex responsible for the internalization of LRP6 *(Binnerts ME et al, 2007; Kim KA et al, 2008)*.

1.6.4.4 Other signaling pathways

1.6.4.4.1 Ephrin signalling pathway

An important way for bone cells to communicate is the Eph-Ephrin system **(Figure 19)**. Osteoclasts express a cell membrane protein called ephrin B2. Interaction of ephrin B2 with its receptor, called EphB4, expressed on osteoblasts triggers a signaling pathway in osteoblasts. This pathway promotes osteogenic differentiation and bone formation: forward signaling.
The unique feature of this pathway is that EphB4, expressed on osteoblasts, can send a message to osteoclasts. Through ephrin B2, a signal is transduced in bone-resorbing cells. This reverse signalling suppresses osteoclast differentiation and blocks bone resorption.
This bidirectional interaction in a cell contact-dependent manner between ephrinB ligands expressed on osteoclasts and Eph receptors on osteoblasts can dynamically enhance the transition from resorption to a reversal phase occurring at each resorption cycle and is an additional control of bone homeostasis.

The Ephrin signaling pathway is important for mediating the anabolic effect of parathyroid hormone on bone. In response to PTH or to PTH-related protein (PTHrP), osteoblasts express multiple proteins. One of them is ephrin B2. This raises the possibility that PTH or PTHrP might regulate ephrinB2 to act in a paracrine or autocrine manner on EphB4 or EphB2. The osteoblast then contributes as a local player to the anabolic action of PTH or PTHrP **(Figure 20)**.

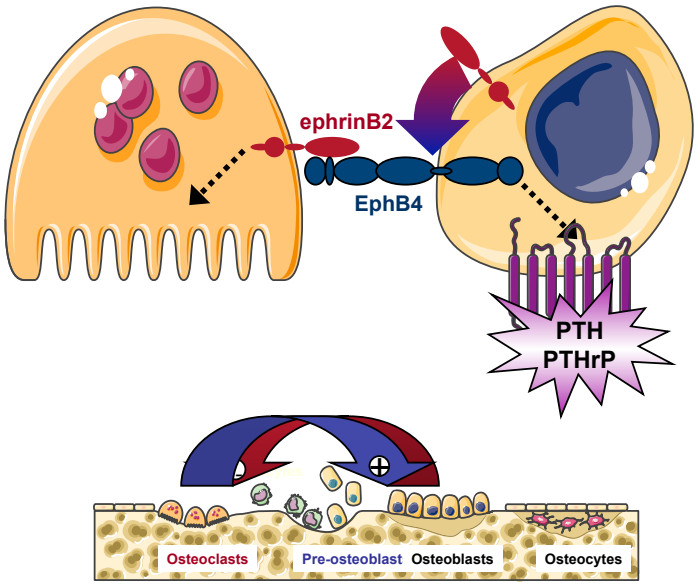


Figure 19: Bidirectional interaction in a cell contact-dependent manner between epiphrin D ligands expressed on osteoclasts and Eph receptor on osteoblasts (Reproduced from Friedlander G, 2011).

1.6.4.4.2 Transcriptional factor : C-Fos

Intermittent PTH needs osteoclasts to stimulate bone formation by osteoblasts. Upon intermittent stimulation by PTH, osteoclasts will mediate pre-osteoblasts proliferation. This proliferation preludes to enhanced bone formation. The effect of PTH on bone formation requires the presence and action of osteoclasts which are alerted by osteoblasts. In animal knock-out models, absence of the transcription factor c-fos results in a lack of osteoclasts and the animals exhibit osteopetrosis. In these animals PTH has no effect on bone formation despite the fact that osteoblast proliferation is still observed. These results illustrate the importance and complexity of the cross-talk between osteoblasts and osteoclasts in bone turnover **(Figure 20).**

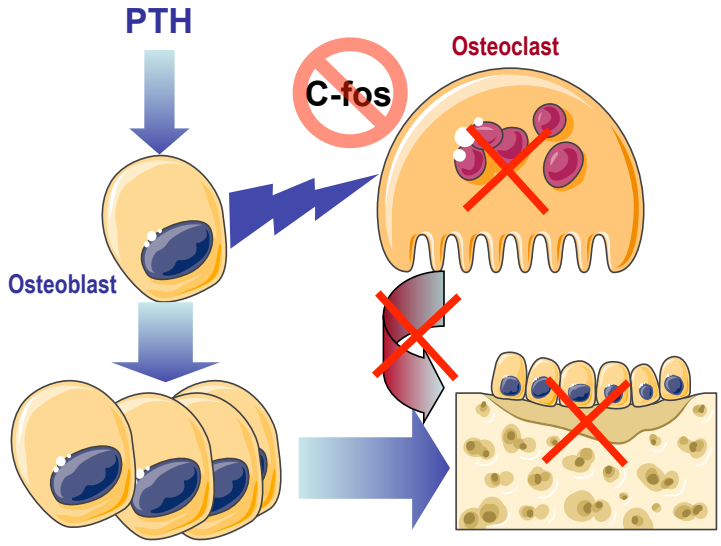


Figure 20: Cross-talk between osteoblast and osteclasts medicated by C-fos (Reproduced from Friedlander G, 2012).

1.7 Pathology of the bone remodeling cycle

1.7.1 Factors affecting the regulatory pathways

Since the 1940s it is known that the bone remodeling, so the proliferation, differentiation and activity of bone cells, is regulated by distant endocrine glands as the gonads and the parathyroid glands *(Khosla S, 2001a; Riggs BL et al, 1998; Rodan GA & Martin TJ, 2000; Manolagas SC, 2000a; Manolagas SC et al, 2002).* At least conceptually, the existence of these two endocrine regulators made the role of other endocrine regulators of bone remodeling most probable *(Karsenty G, 2006; Confavreux CB et al, 2009; Karsenty G & Oury F, 2010)*. Later on, modulations were demonstrated by a variety of other hormones, including growth hormone, 1,25-dihydroxy vitamin D3 and adipokines, and also by numerous other growth factors, including bone morphogenic proteins (BMP's), transforming growth factor β (TGF-β) and cytokines.

In the past 2 decades a series of investigations have started to identify novel and powerfull endocrine modes of regulation of bone mass. These have revealed cellular and molecular bases for the central control of bone mass *(Ducy P et al, 2000b; Takeda S et al, 2002; Elefteriou F et al, 2004; Ahima RS, 2004)*. A number of discoveries are based on the studies of bone physiology in vivo and by analyzing loss-of-function models obtained through homologous recombinations of embryonic stem cells. Examples of this are the discoveries of PTHrP, PTH receptor, receptor activator of nuclear factor-κB ligand (RANKL),

osteoprotegerin (OPG), RUNX2, and C-fos funtions *(Wang ZQ et al, 1992; Karaplis AC et al, 1994; Otto F et al, 1997; Simonet WS et al, 1997; Kong YY et al, 1999)*.

1.7.1.1 Hormones

1.7.1.1.1 Gonadal steroids

Estrogens and androgens influence the development of the skeleton during growth and its maintenance during adulthood. Absence or dysfunction of the sex glands is associated with skeletal abnormalities. The interest in the role of sex steroids on bone was greatly intensified in the 1940s by the original suggestion of Fuller Albright that there was an association between menopause and loss of bone mass *(Reifenstein EC & Albright F, 1947).* Since that time, it has been extensively documented that a deficiency of estrogens in females and both androgens and estrogens in males adversely affects skeletal development and homeostatis during adulthood, and contributes to the development of osteoporosis. The cellular and molecular mechanisms responsible for this adverse effect are still not well understood. Moreover, until recently, it has remained unknown whether and how sex steroid deficiency and old age may influence each other's negative impact on bone.

1.7.1.1.1.1 Hormone biosynthesis of the gonadal steroids

Both estrogens and androgens are derived from C19 metabolites of cholesterol *(Longcope C, 1998; Griffin JE & Wilson JD, 1998)*. Estrogens comprise a large number of molecules, the most abundant of which are 17b-estradiol (E_2), estrone (E_1) and estriol. E_2 is produced in the ovaries and is the main circulating estrogen in premenopausal women **(Figure 21)**.

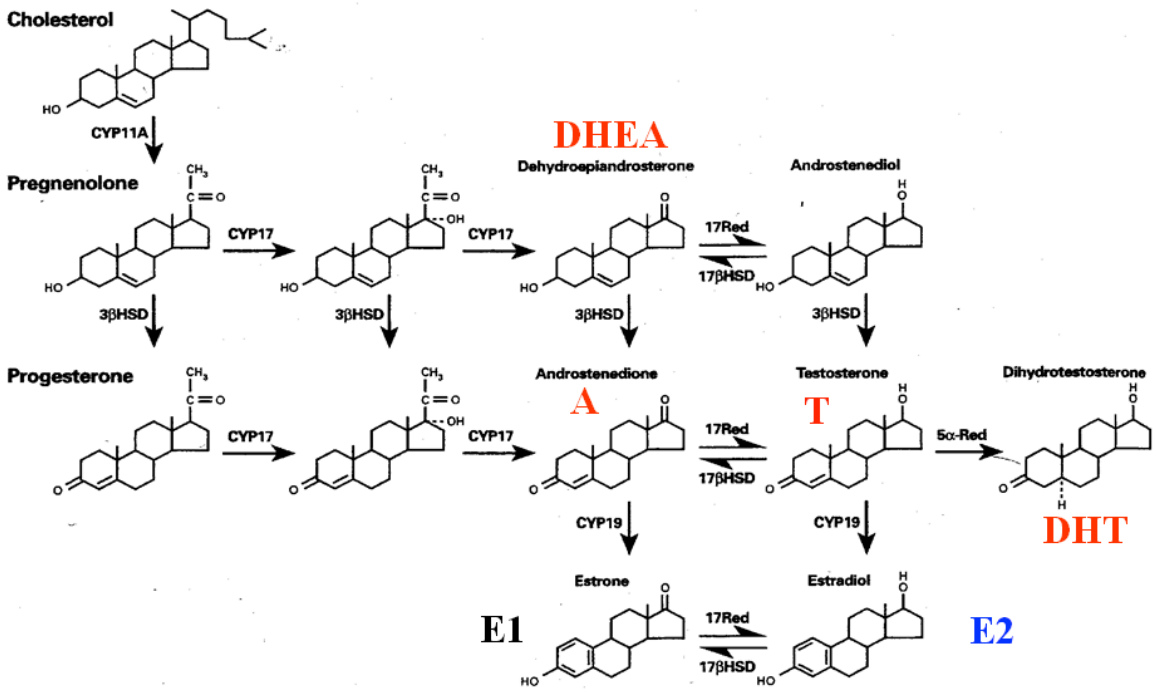


Figure 21: Biosynthesis of estrogens and androgens in the gonads and peripheral tissues principle conversions and major enzyme activities. T= testosterone; E_1=estrone; E_2=17β-estradiol; DHT=dihydrotestosterone; DHEA=dehydroepiandrosterone, HSD=hydroxysteroid dehydrogenase, CYP11A: cytochrome P450 cholesterol side chain cleavage enzyme, CYP17: cytochrome P450 17α hydroxylase/17,20 lyase, CYP19 : aromatase cytochrome P450.

The circulating concentration of E_2 in women is 20-400pg/ml and fluctuates depending on the stage of the ovulatory cycle. The predominant form of estrogens in postmenopausal women is E_1, which is synthesized in several extra-ovarian sites including bone.
The predominant sex steroid in men is testosterone (T), 95% of which is produced by the testes and reaches circulating levels of 3-10ng/ml. The remaining 5% of T originates from the adrenals by the conversion of dehydroepiandrosterone (DHEA) by 3b-hydroxysteroid dehydrogenase (HSD) and 17b-HSD.
Dihydrotestosterone (DHT) is the second most abundant circulating androgen and is provided by the conversion of T by 5α-reductase in peripheral tissues of androgen action. Even though DHT circulates at lower levels than T (0.25-0.75 ng/ml), it has considerably higher affinity for the androgen receptor (AR), and is therefore more potent than T. However, locally produced DHT may be present in higher concentrations in some androgen target tissues, such as in the prostate.
E_2 is also present in the circulation of men (about 10-35 pg/ml) and it is formed by aromatization of T and androstenedion. Approximately 20% originates directly from the testes, while the remaining 80% results from aromatization in peripheral tissues. Estrone (E_1), a markedly weaker estrogen, is produced by aromatization of androstenedione; E_1 can be converted to estradiol by 17b-HSD action **(Figure 21)**.
In women, ovarian C19 steroids intermediates also give rise to T, most of which is then converted to E_2 by P450 aromatase, which is highly expressed in the ovary. The relatively low amonts of T and DHT in women (less than 0.5 ng/ml) are synthesized in the ovary (premenopausal and early postmenopausal women) and in the adrenal cortex (peri- and postmenopausal women).

Fifty to 60% of the total T, and 20-40% of the total E_2, present in the circulation are bound with high affinity to sex hormone binding globuline (SHBG) *(Siiteri PK et al, 1982)*. Most of the remainder is bound nonspecifically to albumin with lower affinity. Of the total circulating levels of either estrogens and androgens only approximately 2% is free to enter the cells and exert biologic effects.

In women after menopause, the circulating levels of estrogens originate from aromatization of adrenal androgens in pheriperal tissues and are generally lower than in men of the same age. The circulating levels of T decrease only marginally (-1%/year) during aging in men, whereas the total E_2 level remains constant. However, SHBG increases markedly in older men, resulting in a greater decrease of the free and bioavailable fractions of T than E_2 *(Khosla S et al, 1998, Kaufman JM et al, 2005)*.

1.7.1.1.1.2 Receptors and molecular mechanisms of action

Similar to other steroid hormones, estrogens or androgens exert their biologic effects by binding to specific receptor proteins : the estrogen receptor α ($ER\alpha$) or b (ER_b) and the androgen receptor (AR), respectively *(Tsai MJ et al, 1994)*. These receptor are ligand-activated transcription factors, that homo- or heterodimerize upon ligand binding and directly attach to specific DNA sequences, called hormone response elements (HREs) in regulatory regions of target genes **(Figure 22)**.

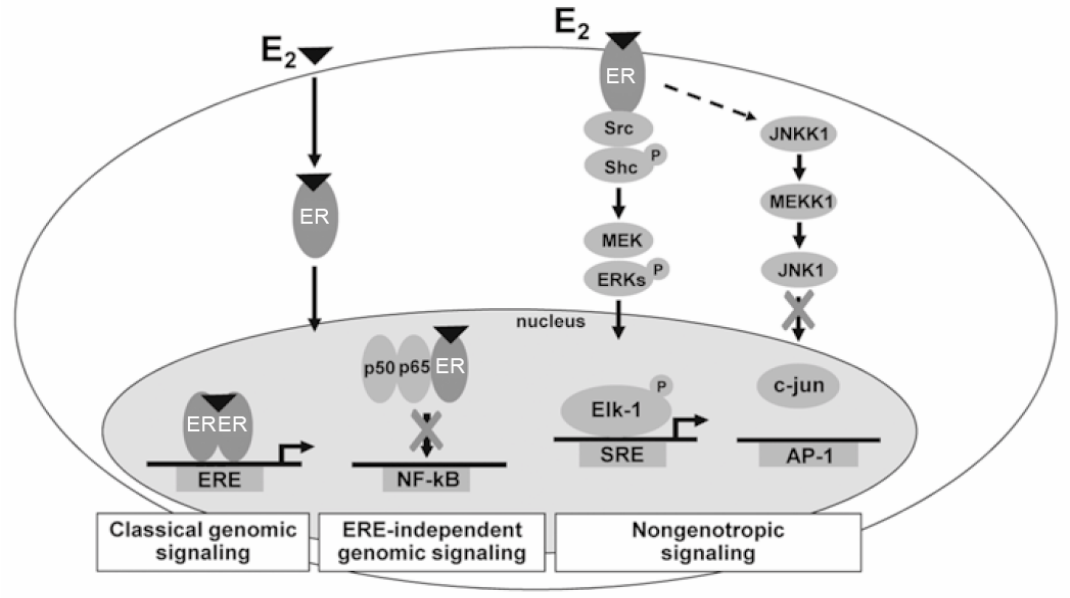


Figure 22: Signaling pathways activated by the estrogen receptor. In the classical genomic signaling pathway 17b-estradiol (E_2 depicted by the triangle) binds to its receptor and translocates into the nucleus where it undergoes dimerization, attaches to estrogen response elements (ERE) on DNA, and activates or represses transcription. In the ERE-independent genomic signaling pathway, the ligand-activated receptor binds to other transcription factors (depicted by the p50 and p60 subunits of NF-κB), thereby preventing them from binding to their reponse element on DNA. In the nongenomic mode of action, the ligand-activated receptor (residing in the plasma membrane or the cytoplasma) interacts with cytoplasmatic kinases and triggers cascades that positively or negatively regulate the activation of transcription factors such as Elk-1 and c-jun (Reproduced from Manolagas SC 2013, Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism; 8th Edition).

DNA-bound receptors interact with several coregulator proteins to form multiprotein complexes that activate or repress the transcriptional machinery *(McKenna NJ et al, 1999)*. Besides direct protein-DNA interactions, steroid receptors are able to interact indirectly with gene promotors through protein-protein interactions with other transcription factors, as is the case of ER binding to nuclear factor-κB (NF-κB). This association inhibits NF-κB activation and the transcription of genes like interleukin 6 (IL-6) *(Stein B & Yang MX, 1995)*.
Estrogens and androgens are also able to evoke nongenotropic actions triggered by the activation of cytoplasmatic signaling cascades, such as Src, Shc, mitogen activated protein (MAP) kinases including extra-cellular regulated kinases (ERKs), phosphatidyl inositol-3kinase (PI3K) and c-jun N-terminal kinases (JNKs).
Such actions result from ligand binding to ERs localized in the cytoplasma or the plasma membrane, followed by downstream kinase-induced changes in the activation of transcription factors *(Kousteni S et al, 2001)*.

ERα, ERβ and the AR have been detected in all skeletal cell types including chondrocytes, bone marrow stromal cells, osteoblasts, osteocytes and osteoclasts and their progenitors *(Vanderschueren D et al, 2004)*. Nevertheless, compared to the reproductive organs, the levels of ER expression in bone cells is low both in males and females; and the ERβ expression is two to three orders of magnitude lower than ERα *(Almeida M et al, 2007)*. The ERβ signaling may not play a major role in bone, with the possible exception that it may exert an inhibitory effect on periosteal apposition *(Windahl SH et al, 1999)*.

1.7.1.1.1.3 Effects of sex steroids on skeletal growth

Sex is an important determinant of the size and shape of the skeleton. Bones grow in length at the growth plate of the epiphyses by the process of endrochondral bone formation. Simultaneously, bones expand radially, their cortices thicken, and the medullary cavities become larger as a result of bone formation at the periosteum and unbalanced remodeling at the endocortical surface – with resorption exceeding formation *(van der Eerden BC et al, 2003)*.
At the initiation of puberty, low levels of estrogens and perhaps of androgens are responsable for a spurt in linear bone growth in both sexes. At the end of puberty, high levels of estrogens are essential for the closure of the epiphyses and the cessation of linear growth. In parallel to the accelaration of linear growth, pubertal boys and girls experience an accelerated enlargement of the outer diameter and further widening of the medullary cavity of long bones. These changes lead to bigger bones in boys than in girls primarily due to a larger increase in periosteal bone formation. In contrasts, girls exhibit more endocortical bone formation than boys. Puberty start earlier in girls, but last longer in boys, without major differences in absolute growth rate. This may account in part for the differences in the size of the skeleton between the two sexes.

Effects on linear growth:
Estrogens are essential for the pubertal bone changes in both girls and boys. Thus, aromatase deficient females and males lacking estrogens do not exhibit a growth spurt and do not undergo closure of the epiphyses *(Jones ME et al, 2007)*. Estrogen replacement in these patients enhances bone growth. Dominant inherited overexpression of the aromatase gene accelerates growth and leads to premature closure of the epiphyses in both sexes *(Stratakis CA et al, 1998)*. The importance of estrogens for the male skeleton is further highlighted by the absence of a pubertal growth spurt in a man with a loss of function mutation of ERα *(Smith EP et al, 1994)*. The stimulatory effect of estrogens on the linear skeletal growth is in part attributed to activation of the GH-IGF axis *(van der Eerden BC et al, 2003)*.
The closure of the growth plate at the end of puberty is clearly mediated by E2 in both men and women as indicated by failure of growth plate closure and continued growth in the man who lacks ERα *(Smith EP et al, 1994)*, and in aromatase-deficient men and women *(Jones ME et al, 2007; Santen RJ et al, 2009)*.
It is not clear whether androgens have a significant effect on linear growth. The first argument suggesting that androgens may not play a significant role is that serum testosterone levels were high in the man with ERα mutation and the aromatase-deficient men. Secondly, men with androgen insensitivity syndrom due to AR mutations have an intermediate height between normal males and females *(Quigley CA et al, 1995)*. On the other hand, administration of DHT in boys with delayed growth, simulated longitudinal growth supporting the role of androgens in this process *(van der Eerden BC et al, 2003)*.

Effects on periosteal expansion:
The greater periosteal expansion in boys as compared to girls during puberty has been ascribed to the higher levels of androgens in the male. Nevertheless, administration of E_2 to an aromatase-deficient young men increased periosteal apposition *(Bouillon R et al, 2004)*. Consistent with this finding, studies in rodents have revealed that both estrogens and androgens, acting via their respective receptors, are involved in expansion of the periosteum in growing males. DHT may be the androgen responsible for the periosteal expansion in the male *(Windahl SH et al, 2011)*. Pharmacological inhibition of E2 synthesis by an aromatase inhibitor reduced periosteal expansion in orchidectomized male mice. In line with the need for both AR and ERα for optimal periosteal expansion, periosteal circumference is smaller in mice that lack both receptors, as compared to mice lacking only one of the receptors *(Callewaert F et al, 2009)*. Unlike the situation in males, estrogens restrain radial bone growth in females, as evidenced by the finding that ovariectomy of growing rats or mice increases periosteal expansion *(Callewaert F et al, 2010a)*. Moreover, female mice that lack ERb exhibit increased periosteal circumferences *(Windahl SH et al, 1999)*. Whether the opposite effect of estrogens on periosteal apposition in males versus females also occurs in humans is unknown.

1.7.1.1.1.4 Effects of sex steroids on skeletal maintenance

Estrogen and androgen deficiency causes loss of bone associated with an increase in the bone remodeling rate, increased osteoclast and osteoblast numbers, and increased resorption and formation, albeit unbalanced. Conversely, estrogens or androgens decrease bone resorption, restrain the rate of bone

remodeling, and help to maintain a focal balance between bone formation and resorption. These effects are evidently the result of hormonal influences on the birth rate of osteoclast and osteoblast progenitors in the bone marrow as well as of the pro-apoptotic effects on osteclasts and anti-apoptotic effects on mature osteblasts and ostecytes. This may be the result of direct effects of estrogens or androgens and/or indirect effects mediated by cytokines (including IL-1b, IL-6, IL-7, TNFα, M-CSF, RANKL, OPG and prostaglandins) produced by the bone marrow stromal cells, T and B lymphocytes, macrophages, and dendritic cells *(Manolagas SC et al, 2002; Weitzmann MN & Pacifici R, 2006)*.

Effects on osteoclasts:

Selective deletion of the ERα from osteoclasts has demonstrated that cell autonomous effects of estrogens on osteoclasts mediated via the ERα account for most, if not all, the antiresorptive properties of estrogens on the female skeleton *(Nakamura T et al, 2007; Martin-Millan M et al, 2010)*.

Effects on osteoblasts:

Elimination of the pro-apototic effects of estrogens on osteoclasts is sufficient for loss of bone in the cancellous compartement, in which complete perforation of trabeculae by osteoclastic resorption precludes subsequent refilling of the cavities by the bone forming osteoblast. The effects of estrogens on osteoblasts, and perhaps other cell types, are indispensable for their protective effects on the cortical compartment.
ERα promotes proliferation/differentiation and/or survival of osteoblast progenitors in both sexes *(Manolagas SC & Parfitt AM, 2010a)* **(Figure 23).**

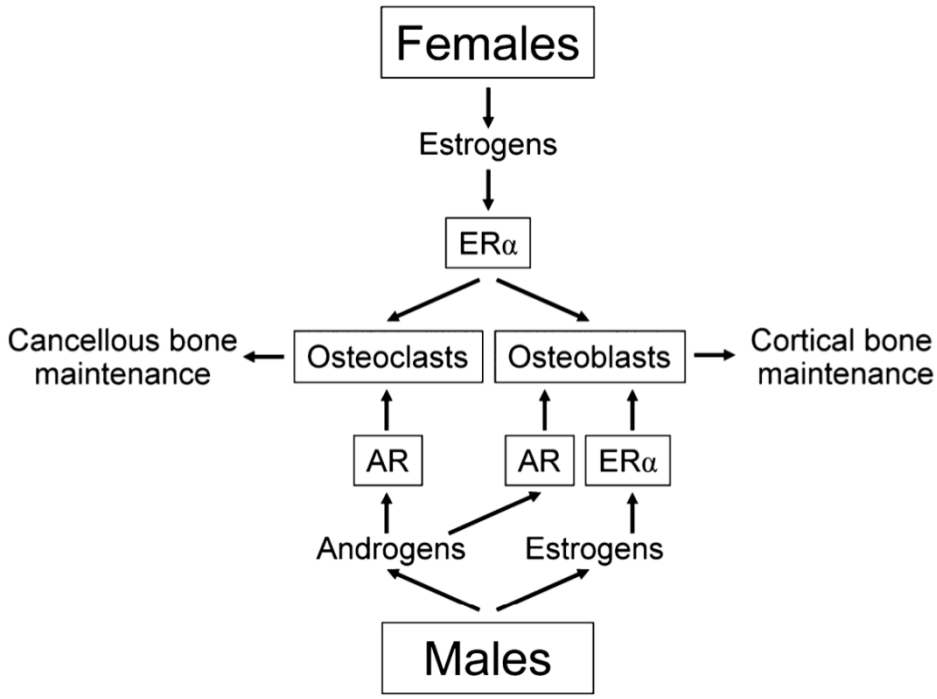


Figure 23: Protective effects of estrogen and androgen on the female vs the male skeleton. In the females, estrogens protect against loss of cancellous and cortical bone by cell autonomous effects on osteoclasts and osteblasts, respectively. In males, cell autonomous effects of androgens (acting via the AR) on osteclasts are responsible for the protection of the cancellous compartment. However, cell autonomous effects of both androgens and aromatizable androgens converted to estrogens are responsible for the protection of the cortical bone compartment (Reproduced from Manolagas SC 2013, Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism; 8th Edition).

Effects on osteocytes:

Osteocytes are one of the cellular targets of estrogen and androgen action. Estrogen or androgen inhibit osteblast and osteocyte apoptosis, and this effect requires activation of the Src/Shc/ERK signaling pathway and downstream trancription factors *(Kousteni S et al, 2001; Kousteni S et al, 2002)*. Conversely, estrogen and androgen deficiency increases osteocyte apoptosis in humans *(Tomkinson A et al, 1997)*, rats *(Tomkinson A et al, 1998)*, and mice *(Kousteni S et al, 2002)*.
Harald Frost hypothesized that mechanical strain, perceived by a hypothetical skeletal mechanostat, leads to changes in bone remodeling in order to adjust bone mass to a level that is appropriate to the current ambient mechanical forces *(Frost HM, 1987)*.
Estrogens are thought to decrease the minimum effective strain necessary to inititate bone formation. In support of this hypothesis, estrogens and exercise may exert additive effect on bone mass in humans *(Marcus R, 2002)* and mice *(Lee K et al, 2003)*. Physiological levels of mechanical strain prevents apoptosis of osteocytic cells *(Plotkin LI et al, 2005)*. The evidence of a role of ER in the pro-survival effect of mechanical strain on osteocytes is consistent with the poor osteogenic response to loading exhibited by mice lacking ERα or ERb *(Lee K et al, 2003)*.

1.7.1.1.1.5 Loss of sex steroids and aging

For over 60 years, skeletal involution with advancing age has been attributed primarily to the decline of ovarian function at menopause, and to a later and smaller decline of estrogens in older males *(Manolagas SC, 2010b; Khosla S et al, 2011)*. However, recent epidemiologic evidence in humans has made it clear that the balance between bone formation and resorption becomes progressively negative with advancing age in both women and men prior to, and independently from, any decline in sex steroids. Furthermore, in a study by Recker et al *(Recker R et al, 2000),* extrapolation of the changes in bone mass found in perimenopausal women before the menopausal estrogen drop and after its completion indicates that between menopause and the age of 75, women approximately lose 22% of their total body bone mineral; of this, 13,3% is due to aging and 7.7% to estrogen deprivation *(Recker R et al, 2000)*. In addition, recent analysis of cortical bone loss with high resolution CT of the radius and of postmortem femurs of women age 50 to 80 years has revealed that most of the bone loss in the old age is the result of increased cortical porosity *(Zebaze RM et al, 2010)*. Studies have suggested that while the onset of cortical bone loss in humans is closely tied to estrogen deficiency, a significant proportion of trabecular bone loss is estrogen-independent *(Khosla S et al, 2011)*.

Reactive oxygen species (ROS) influence the birth and death of bone cells:

Oxidative stress (OS) has been thought for many years to be a common mechanism in the pathogenesis of several degenerative disorders associated with aging. The same may hold true for osteoporosis. An increase of reactive oxygen species (ROS) may be implicated in the decrease of bone formation associated with aging, as well as in the increase of bone resorption associated with estrogen deficiency *(Almeida M et al, 2007; Manolagas SC, 2010b; Lean JM et al, 2003)*. Increased ROS production in osteoblasts stimulates apoptosis *(Almeida M et al, 2007; Ambrogini E et al, 2010; Jilka RL et al, 2010)*. ROS are a critical requirement for RANKL-induced osteoclast generation, activation and survival **(Figure 24)**. It is widely recognized that ROS can be both harmful byproducts of aerobic metabolism that damage proteins, lipids and DNA leading to cell demise, as well as signalers produced by cells for the purpose of propagating intracellular signaling from cell surface receptors *(Manolagas SC, 2010b)*.

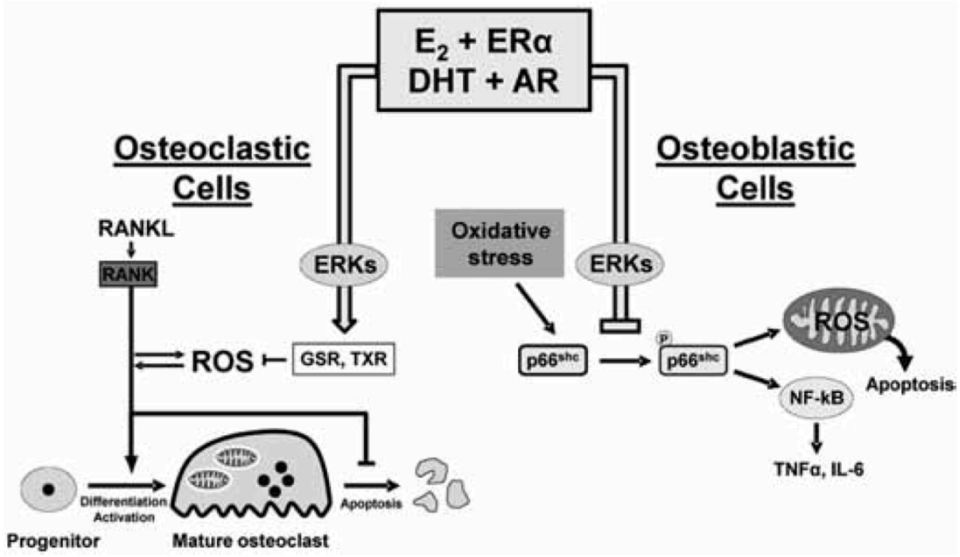


Figure 24 : Reactive oxygen species (ROS) influence the birth and death of osteclasts and osteoblasts, and their effect are counterregulated by sex steroids. In osteoclasts, ROS are required for the RANKL-induced differentiation, activation and survival. On the other hand, ROS stimulate osteoblast apoptosis and attenuate osteblastogenesis. Estrogens and androgens, acting via cytoplasmatic kinases like ERKs, antagonize the production or the effects of ROS. These antioxidant actions are responsible for the ability of sex steroids to attenuate osteoclast generation and survival, promote osteoblast and osteocyte survival, and attenuate NF-kB and cytokine production (Reproduced from Manolagas SC, 2010b).

To protect against OS, organisms scavenge ROS by a network of overlapping mechanisms, including various forms of superoxide dismutases (SODs) and catalase as well as thiol-containing oligopeptides with redox-active sulfhydryl moeities. Reduction of these scavenging mechanisms along with the mitochondrial respiratory chain leakage and increased activity of oxidases in other cellular compartments are the three main mechanisms of OS *(Manolagas SC, 2010b)*.
Recently, foxO transcription factors have emerged as another important defense mechanism against OS, serving primarily to maintain the integrity of long-lived cells, including stem cells *(Ambrogini E et al, 2010)*.

Estrogens and androgens attenuate oxidative stress in bone cells:

Sex hormone deficiency, similar to aging, increases the generation of ROS. Some of the adverse effects of acute loss of ovarian or testicular function on bone might be prevented by anti-oxidants *(Almeida M et al, 2007; Lean JM et al, 2003).* Sex steroid deficiency accelerates the effects of aging *(Manolagas SC & Parfitt AM, 2010a; Manolagas SC, 2010b).* Estrogens or nonaromatizable androgens decrease OS and antagonize ROS-induced osteoblast apoptosis, NF-kB activation, and cytokine production in osteoblastic cells *(Almeida M et al, 2007; Almeida M et al, 2010)*.

1.7.1.1.1.6 Sex steroids deficiency and the development of osteoporosis

Both women and men lose bone as a result of aging, but men are less likely to develop osteoporosis than women for two reasons. First, men gain more bone during puberty, and second they lose less bone during aging because unlike women, men do not experience an abrupt loss of estrogens.
The accelerated cancellous bone loss and decrease of bone strength caused by menopause results predominantly from trabecular perforation and loss of connectivity. This phase is followed a few years later by a phase of slower bone loss that primarily affects cortical bone sites. This later stage occurs in both women and men and is associated with a decrease in osteoblast number and bone formation rate. In

line with this, decreased wall width is the most consistent histologic finding in older women and men with osteoporosis. Moreover, in older men bone loss is associated with trabecular thinning rather than perforation *(Ebeling PR, 2008)*.

Estrogens versus androgen deficiency in men with osteoporosis:

Estrogen deficiency may be an important mechanism of the development of osteoporosis not only in females but also in males. Support for his view is provided from three types of evidence: (1) the genetic evidence from men with ERα or aromatase mutations discussed earlier *(Santen R et al, 2009)*; (2) results of short-term clinical experimentation with administration of aromatase inhibitors *(Santen R et al, 2009)*; and (3) cross-sectional correlations between free serum estradiol levels and BMD or bone remodeling markers *(Khosla S et al, 2011)*. Furthermore, it remains unclear whether, and to what extent, small differences in estrogen or androgen levels in the circulation contribute to changes in bone markers or BMD in older men, especially as similar small differences do not seem to cause BMD differences in pre- or perimenopausal women *(Sowers MR et al, 2003)*.

On the other hand, the important role of androgens and the androgen receptor (AR) in the homeostasis of the male skeleton in humans is well illustrated by the low bone mass of men with idiopathic hypogonadotropic hypogonadism or complete androgen insensitivity syndrome *(Finkelstein JS et al, 1987; Marcus R et al, 2000)*.

From the most recent animal studies on AR and ERα global or cell specific knock out models, the current understanding is unclear about how much of the bone-sparing effect of androgens on the adult male skeleton results from effects of T or DHT acting via the AR as opposed to androgens converted to estrogens and acting via the ERα *(Callewaert F et al, 2010b)*. It remains also unclear to what extent the bone-sparing effect of androgen results from cell autonomous actions on osteclasts, osteoblasts or some other cell types, and whether their protective effects on the adult skeleton results from similar or different mechanisms from those responsible for their effects on skeletal growth. Thus, a considerable gap in the understanding of the basic and clinical aspects of androgen action on bone makes it uncertain in how far of the osteoporosis in old men results from sex steroid deficiency or from aging. Considering the hormonal component, the question remains how much is due to true estrogen versus androgen deficiency and/or a decrease in bioavailable E_2 as opposed to T or DHT. However, several clinical studies show a correlation between a decrease in bioavailable E2, but not T, and bone mass in older men *(Khosla S et al, 2011)*.

1.7.1.1.1.7 Role of sex steroids in the treatment of osteoporosis

The recognition of serious side effects associated with natural estrogen-based therapies in the uterus, breast, and the cardiovascular system, as well as their decreased efficacy in older postmenopausal women, has significantly reduced the prolonged use of estrogen replacement for the prevention or treatment of osteoporosis *(Rossouw JE et al, 2002; North American Menopause Society, 2010)*. The selective estrogen receptor modulator, raloxifene, obviates the adverse effects of estrogens on the uterus and breast, and is effective in reducing fracture risk in postmenopausal women with osteoporosis. However, its efficacy is lower than that of natural estrogens and other alternative antiresorptive agents, such as bisphosphonates and the anti-RANKL antibody denosumab.
Testosterone reduces bone turnover and increases bone mass in hypogonadal men, but evidence of a protective effect of testosterone treatment on fracture risk is men is not available.
Selective AR modulators (SARMs) have been shown in preclinical studies to retain the anabolic efficacy of androgens on bone and muscle while acting as partial antagonists in the prostate *(Rosen J & Negro-Vilar A, 2002)*. The development of SARMs as therapeutics, has been hindered by the evidence that they may have adverse effects on the heart. Therefore, the future of such compounds is at this stage uncertain.

1.7.1.1.2 Vitamin D

Once considered just a vitamin important for bone health, vitamin D has been rediscovered as a pleiotropic hormone during the last three decades *(Verstuyf A et al, 2010)*. By regulating the expression of 3% of the genome, vitamin D affects the functions of most cell types *(Heaney RP, 2008; Norman AW, 2008)*.

After being synthesized in the skin (cholecalciferol) or ingested with supplements or certain foods (both ergocalciferol and cholecalciferol), vitamin D is hydroxylated to 25-hydroxyvitamin D (25(OH)D) by cytochrome P-450-dependent enzymes *(Heaney RP, 2008; Norman AW, 2008; Henry HL, 2011)*. Although there is evidence of some 25-hydroxylation in other organs, the liver accounts for most of the 25(OH)D production. Another cytochrome P-450-dependent hydroxylase, CYP27B1 or 1α-hydroxylase, catalyses the conversion of 25(OH)D into 1,25 dihydroxyvitamin D ($1,25(OH)_2D$ or calcitriol), the primary hormonally active form of vitamin D *(Norman AW , 2008; Henry HL, 2011)*. By binding to and activating the nuclear vitamin D receptor (VDR), $1,25(OH)_2D$ modulates the transcription of thousands of genes. The retinoid X receptor and several cofactors cooperate with the $1,25(OH)_2D$-VDR complex to regulate gene expression *(Norman AW , 2006)*. Some vitamin D effects occur via nongenomic responses by a VDR associated with the plasma membrane in certain cells *(Norman AW, 2008)*.

The 1α-hydroxylation reaction is the point at which the vitamin D system branches in two **(Figure 25)**

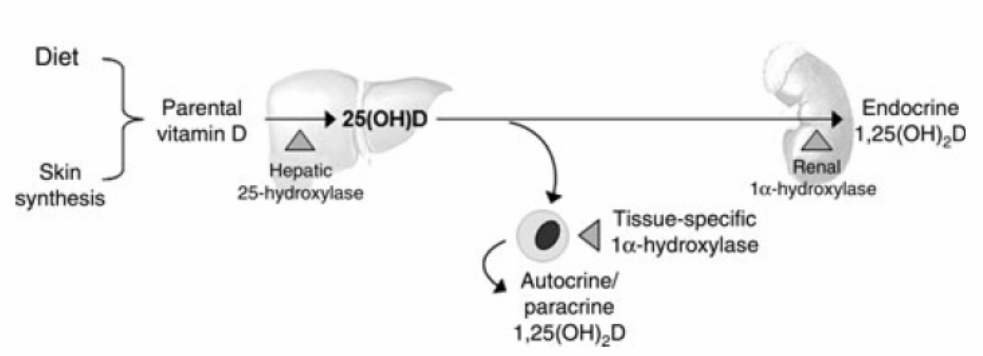


Figure 25: The vitamin D system. Vitamin D is produced in the skin or ingested with food. To be activated, it is hydroxylated twice: first to 25-hydrovitamin D (25(OH)D) and than to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). While the liver accounts for most25-hydroxylation, the 1α-hydroxylation reaction occurs in virtually any tissue of the body. In physiological conditions, however, only the $1,25(OH)_2D$ produced by the kidney enters the circulation to act in an endocrine way. The hormone synthesis in extrarenal sites, functions in an autocrine and paracrine manner (Reproduced from Ameri P et al, 2013).

The renal tubular cells produce $1,25(OH)_2D$ from 25(OH)D supplied by the vasculature. Most $1,25(OH)_2D$ synthesized in the kidney leaves the organ and, by acting in a classical endocrine way, participates in bone metabolism and stimulates intestinal absorption of calcium and phosphate. Furthermore, it inhibits PTH release from the parathyroid glands *(Heaney RP, 2008; Norman AW, 2008; Verstuyf A et al, 2010).*
Renal 1α-hydroxylase, and so the endocrine actions of vitamin D, are tightly regulated by phosphate, PTH and fibroblast growth factor 23 (FGF23). FGF23 is principally secreted by the osteocytes and is a potent inhibitor of the 1α-hydroxylation of 25(OH)D in the kidney *(Donate-Correa et al, 2012)*. Excess phosphate is the major stimulus for FGF23 production, which inhibits renal phosphate reabsorption.
It is now believed that the bulk of circulating 25(OH)D is taken up by extra-renal 1α-hydroxylase-expressing tissues, which make their own 1,25(OH)2D for paracrine and autocrine activity *(Zehnder D et al, 2001; Verstuyf A et al, 2010)*. Extra-renal 1α-hydroxylase is principally regulated in a tissue-specific manner by several factors *(Henry HL, 2011)*, such as cytokines *(Liu PT et al, 2006)*. In general, peripheral production of $1,25(OH)_2D$ accounts for many of the extra-skeletal effects of vitamin D. These effects would otherwise be difficult to explain based on vitamin D endocrine actions alone *(Heaney RP, 2008; Norman AW, 2008)*.
In normal conditions, locally produced $1,25(OH)_2D$ is used rapidly and does not leak into the circulation *(Heaney RP, 2008)*. Hence, serum $1,25(OH)_2D$ levels are only a measure of renal-derived vitamin D. In contrast, the concentration of 25(OH)D is a good indicator of the amount of vitamin D globally available for both endocrine and paracrine/autocrine functions.

The local action of $1,25(OH)_2D$ and VDR signaling in bone is not essential when sufficient dietary calcium is absorbed *(Heaney RP, 2008; Norman AW, 2008)*. Hypocalcemia or decreased intestinal calcium absorption leads to increased parathyroid hormone (PTH) secretion, which stimulates renal calcium reabsorption and bone resorption. PTH also enhances production of the active form of vitamin D, $1,25(OH)_2D$ which in turn activates the VDR in the intestine to increase calcium absorption and in the

bone to induce bone resorption *(Verstuyf A et al, 2010)*. Elevated $1,25(OH)_2D$ levels do not only stimulate bone turnover potentially leading to osteopenia, but also suppress bone matrix mineralisation. This leads to extensive hyperosteoidosis and hypomineralization of the bone cortex, which in turn contributes to the increase of bone fractures.
Mechanistically, it has been postulated that osteoblastic VDR signaling suppresses calcium incorporation in bone by directly stimulating the transcription of genes encoding mineralization inhibitors, e.g. osteopontin *(Meyer MB et al, 2010)* and pyrophosphate (PPi) *(Lieben L et al, 2012)*. Studies indicate that maintainig normocalcemia has priority over skeletal integrity. In this respect $1,25(OH)_2D$ minimizes skeletal calcium storage, not only by increasing calcium release from bone, but also by inhibiting calcium incorporation into bone *(Lieben L et al, 2012)*.

1.7.1.1.3 Parathormone and PTHrP

Mammalian parathormone (PTH) is synthetized as a pre-pro-peptide comprising 115 aminoacids, but only the single chain 84 full length polypeptide is secreted by the parathyroid glands. PTH displays considerable sequence homology with PTH-related peptide (PTHrP) *(Karaplis AC, 1994)*, which is, however, limited to the amino-terminal 1-34 region. PTH-rP, originally discovered as the hormonal mediator of hypercalcemia of malignancy *(Strewler GJ et al, 1987)*, plays a physiological role in the regulation endochondral bone formation, smooth muscle function, and branching morphogenesis of the mammary gland *(Gensure MC et al, 2005)*.
The parathyroid glands first appeared during evolution with the movement of animals from an aquatic environment to a terrestrial environment deficient in calcium. Maintenance of adequate levels of blood ionized calcium is required for normal neuromuscular function, bone mineralization, and many other physiological processes.
The major physiological function of parathyroid glands is to act as a "calciostat", sensing the prevailing blood inonized calcium level and adjusting the secretion accordingly.
Small decrements in serum ionized calcium result in secretion of PTH. PTH restores normocalcemia by promoting bone resorption and releasing calcium from the skeletal reservoir, by reducing urinary calcium losses and increasing phosphate excretion, and by enhancing intestinal calcium absorption, indirectly through the renal production of the active vitamin D metabolite $1,25(OH)_2$ vitamin D. The blood ionized calcium and $1,25(OH)_2$ vitamin D contribute to the negative feedback inhibition of PTH excretion, whereas serum phosphate increases PTH secretion.
The relationship between the ionized calcium and PTH secretion is a steep sigmoidal one, allowing significant PTH secretion in response to very small changes in blood ionized calcium. The midpoint of this curve ("set point") is a refletion of the sensitivity of the parathyroid gland to suppression by extracellular calcium *(Brown EM, 1983)*.
Extracellular calcium is "measured" at the surface of the parathyroid cell through a calcium-sensing receptor (CaSR) that is abundantly expressed at the plasmamembrane of these cells *(Brown EM, 1993)*. Increased levels of extracellular calcium suppress PTH secretion, while diminished levels increase PTH secretion.
Under normal physiologic condition, there is minimal proliferation of parathyroid cells. However, chronic hypocalcemia elicits an increase in both the size and number of the parathyroid cells *(Cozzolino M et al, 2005)*. It is of considerable interest that CaSR is also expressed in a number of other tissues. Although the physiological role of the CaSR in other peripheral tissues is not well understood, recent studies with conditional knockout models, suggest that the expression of CaSR in chondrocytes and osteoblasts is essential for normal endochondral bone formation *(Chang W et al, 2008)*.

Beside the actions of PTH to stimulate bone resorption, more recent data illustrate the osteogenic effects of the peptide hormone. The anabolic effect of intermittent PTH administration is preserved in the absence of LRP5. These findings are in favour of distinct signaling pathways involved in the effects of PTH and Wnt/Lrp5 on bone formation (see **Figure 16** for the canonical Wnt/b-catenin pathway and **Figure 19** for the Eprhin and signaling pathway).

1.7.1.1.4 FGF23

Bone itself is an endocrine organ, which secretes circulating factors and hormones. One of these is fibroblast growth factor 23 (FGF23), which is synthesized by osteoblasts and osteocytes. Its secretion is

dependent on the phosphate level in the extracellular fluid. FGF23 acts on the kidney where it promotes phosphaturia by inhibiting phosphate transport in the proximal tubule and where it acts to lower $1,25(OH)_2D$ production, thereby decreasing intestinal calcium absorption **(Figure 26)**. More recently it has been shown, that FGF23 is also an auto and paracrine factor acting on bone-forming cells. Overexpression of FGF23 by bone-forming cells inhibits osteoblast differentiation and matrix mineralization. Animal studies have highlighted more complex interactions between PTH and FGF23 *(Friedlander G, 2012)*.

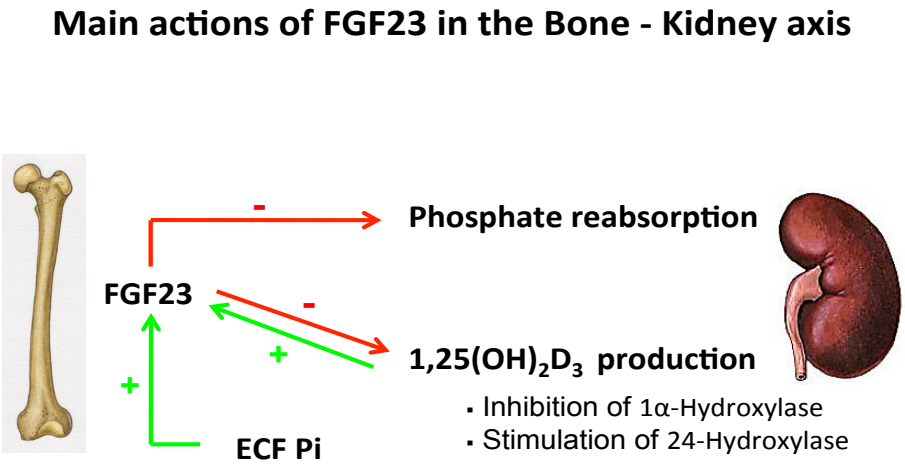


Figure 26: Physiology of FGF23 in the regulation of phosphate homeostatis

1.7.1.1.5 Serotonin

Serotonin is synthesized in two steps from the essential amino acid tryptophan with the rate limiting step being catalysed by the enzyme tryptophan hydroxylase. The brain and the intestine are the two major sites of serotonin synthesis. Gut-derived serotonin (GDS) has been recently shown to inhibit bone formation by reducing osteoblast proliferation.
There is a tryptophan hydroxylase inhibitor, which specifically inhibits gut-derived serotonin synthesis. Administration of this inhibitor to osteoporotic rodents resulted in increased osteoblast proliferation and bone mass *(Yadav VK et al, 2010; Cui Y et al, 2011).* This inhibition of gut-derived serotonin synthesis may potentially be a useful future treatment of osteoporosis, if the role of serotonine is confirmed in humans.
LRP5-deficient mice have a decreased bone mass. A recent theory about this effect is the inhibition of serotonin by LRP5 in enterochromaffin cells of the duodenum, secondary to inhibition of tryptophan hydroxylase 1.
Serotonin synthesized in the duodenum is released in the circulation. Serotonin reaches the bone where it binds to a specific receptor: 5-hydroxytryptamine (serotonine) receptor 1B (Htr1b) on osteoblasts. Through this receptor interaction and downstream events osteoblast proliferation is inhibited *(Yadav VK et al, 2010; Cui Y et al, 2011)*.
Loss-of-function mutations in LRP5 will lead to increased levels of circulating serotonin and a low bone mass phenotype. LRP5 gain-of-function mutation results in high bone mass phenotypes by decreasing serotonin levels *(Saarinen A et al, 2010; Frost M et al, 2010)*. This contrasting concept with the already existing extensive evidence pointing towards a direct effect of LRP5 on osteoblastic cell through Wnt/β-catenin signaling, was challenged in further studies. These studies could not confirm the serotonine level changes *(Cui Y et al, 2011)*. So the exact mechanism whereby LRP5 acts on the bone needs to be elucidated in further studies *(Goltzman D, 2011)*.

1.7.1.1.6 Growth hormone

1.7.1.1.6.1 General introduction

Growth hormone (GH) and insuline-like growth factor I (IGF-I) are important regulators of bone hemostasis throughout life *(Ohlsson C et al, 1998)*. During embryonic development IGF-I and IGF-II are key determinants of growth, acting independently of GH *(Woods KA et al, 1996)*. During the prepubertal and pubertal period, GH and IGF-I are determinants of longitudinal bone growth, skeletal maturation and the acquisition of bone mass, whereas in adults they are important in the maintenance of bone mass *(Monson JP et al, 2002; Baroncelli GI et al, 2003)*.

The process of longitudinal bone growth at the cartilagenous growth plate is regulated by genetic and endo/paracrine hormonal factors, the cellular environment and nutrition *(Nilsson O et al, 2005)*. In addition to the effects on longitudinal growth, GH and IGF-I have potential effects on bone modeling and remodeling. In contrast to bone remodeling, as decribed above, bone modeling is a process of uncoupled bone formation and bone resorption *(Parfitt AM, 2001; Canalis E, 2005)*. Bone modeling is in many situations regulated by mechanical forces and it serves to maintain bone shape and mass. GH and IFG-I exert their anabolic effects on trabecular and cortical bone. The latter occurs by periosteal bone apposition, a process of matrix deposition at the outer surface of bone, resulting in increased bone width, and skeletal strength *(Seeman E, 2003)*. Effects on the periosteal apposition by GH and IGF-I may explain the characteristic bone deformities occuring in acromegaly. The anabolic effects of GH and IGF-I are important for the acquisition of bone mass during adolescence and possibly for the maintenance of skeletal architecture during adult life. Late adolescence and early adulthood are critical periods for the achievement of peak bone mass *(Bangor JP et al, 1991; Theintz G et al, 1992; Matkovic V et al, 1994)*. This is a critical determinant of future risk of osteoporosis. The precise time of the attainment of peak bone mass is not defined with certainty and is skeletal-site dependent. Important interaction between the gonadal steroids and growth hormone axis occur. The increase of gonadal steroid synthesis during puberty is an important hormonal regulator of bone accretion. Boys with constitutionally delayed puberty achieve lower peak bone mass than normal boys *(Woods KA et al, 1996)*.

In addition to changes in sex steroid levels that occur with aging in both men and women, sex steroid-independent factors including in the production of growth factors important for osteoblast differentiation and function also occur. With aging, both the frequency and amplitude of growth hormone secretion is diminished *(Ho KY et al, 1987)*, leading to decreased hepatic production of IGF-1 and IGF-2, an effect that may contribute to decreased bone formation with aging *(Boonen S et al, 1999)*. Additionally, aging is associated with increased levels of the IGF inhibitory binding protein, IGF-BP2, which also correlates inversely with bone mass in older subjects *(Amin S et al, 2004)*.
It is likely that independently of changes in sex steroids and other hormonal factors, intrinsic changes in osteoblast and osteoclast lineage cells occur as a consequence of the aging process. Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stem cells *(Moerman EJ et al, 2004)*.

1.7.1.1.6.2 Growth hormone (somatotropin)

GH is a single-chain peptide of 191 amino acids. GH was isolated from somatotrophs, cells of the anterior pituitary gland *(Raben MS, 1958)*. The synthesis and release of GH is under control of central and peripheral signals and subject of multiple feedback mechanisms. The pituitary growth hormone releasing hormone (GHRH) promotes GH release, which in turn is inhibited by somatostatin (GH inhibiting Hormone (GHIH)) or Somatotropin releasing inhibitory Hormone (SRIH)) *(Giustina A et al, 1998)*.
IGF-I, which is secreted by the liver under GH control, also inhibits GH secretion in somatotrophs. In addition IGF-I indirectly inhibits GH release by stimulating the release of somatostatin *(Giustina A et al, 1998)*.

Ghrelin, a 28-amino acid peptide synthesized by the gastrointestinal tract, is an endogenous inducer of GH release *(Takaya K et al, 2000)*. Finally, GH secretion is under influence of additional hormonal signals, and

sex steroids and thyroid hormone stimulate, whereas glucocorticoids inhibit, GH secretion *(Wehrenberg WB et al, 1992; Giustina A et al, 1992; Giustina A et al, 1995; Giustina A et al, 1998)*.

GH circulates bound to a GH-binding protein, which is the extracellular domain of the GH receptor (GHR) *(Clark RG et al, 1996)*. GH-binding protein is synthesized primarily by the liver, although synthesis by extrahepatic tissues, such as muscle and adipose tissue, may contribute to the circulating levels of GH-binding protein *(Fisker S et al, 1997; Ballesteros M et al, 2000)*. The function of GH-binding protein is not completely understood, although it may modulate the activity of GH either by prolonging its half-live or by reducing its availability to the GHR. The GHR is highly expressed in the liver, adipose tissue, heart, kidneys, intestine, lung, pancreas, cartilage, and skeletal muscle.

Serum GH levels decline with age, reaching a lowest level at the sixth decade *(Zadik Z et al, 1985)*. In aged men, the daily GH secretion is 1/5 to 1/20 of that observed in young adults *(Veldhuis JD et al, 2003)*. The GH output decreases twice as rapidly in men compared to women *(van den Berg G et al, 1996)*. The age-dependent decline in GH secretion is secondary to a decrease in GHRH and to an increase in somatostatin secretion *(Giustina A et al, 1998)*. These change occur at the hypothalamic level, but their cause is unknown. A reduction in central cholinergic tone leading to an increased somatostatin release possibly explains the change in GH secretion *(Coiro V et al, 2002)*. The decline in sex steroids, physical activity, and the presence of aberrant sleep patterns also may contribute to the decline of GH levels during aging *(Veldhuis JD, 1996)*.

1.7.1.1.6.3 Insuline-like growth factors (IGFs)

GH acts by inducing the synthesis of IGF-I in the liver. However, the physiology of IGF-I is complex because it acts both as a circulating hormone and as a local growth factor *(Melmed S, 1999)*. IGF-I is also synthesized in multiple extrahepatic tissues, (including cartilage and bone), where it acts as a local growth factor under the control of diverse hormones.
Locally, the availability and activity of IGF-I also is regulated by IGFBPs, and in vitro studies have demonstrated that at the tissue level most of the IGF is bound IGFBPs, with only a small fraction present in the unbound free form. The cellular actions of IGF-I are mediated by an IGF-I receptor (IGF-IR), a receptor tyrosine kinase expressed in IGF target tissues *(Adams TE et al, 2000)*.
IGF-II shares biochemical and biological properties with IGF-I and it is important in skeletal development, but its function in the adult skeleton is not proven. IGF-II is synthesized by skeletal cells. Its synthesis is not GH dependent *(Collett-Solberg PF & Cohen P, 2000)*.
For more information on IGFs see paragraph 1.7.1.3.

1.7.1.1.7 Thyroid hormone

Aside from the regulation of bone mass by the gonadal and somatotropic axes *(Lapauw BM et al, 2009a; Lapauw BM et al, 2009b)* an other determinant of bone mass is thyroid hormone. Thyroid hormone is essential for normal bone growth, maturation and metabolism. Its deficiency in children results in delayed skeletal development, delayed bone age and growth arrest accompanied by epiphyseal dysgenesis *(Murphy E & Williams GR, 2004)*.
In adults with hypothyroidism, bone turnover is reduced with a prolonged bone formation phase that leads to an increased mineralization phase and an apparent increase of bone mineral density (BMD). Hyperthyroidism in adulthood is associated with increased bone turnover and a reduction in BMD due to increased cortical porosity and accelerated bone loss *(Eriksen EF, 1986; Mosekilde L et al, 1990; Vestergaard P & Mosekilde L, 2003; Lakatos P, 2003; Karga H et al, 2004)*.

Both hypo- and hyperthyroidism in adults have been associated with increased fracture risk *(Vestergaard P & Mosekilde L, 2002)*. In a study of male siblings at the age of peak bone mass (PBM) we recently demonstrated that between subject variation of thyroid hormones within physiological ranges has an effect on bone mass, density and geometry. Higher levels were associated with lower areal BMD and BMC at various skeletal sites *(Roef G et al, 2011)*.

1.7.1.2 **Growth factors**

1.7.1.2.1 Transforming growth factor β (TGFβ)

TGFβ is a critical regulator of bone homeostasis (see also paragraph 1.7.1.4.3). Through its effects on osteoblast and osteoclast migration, proliferation, differentiation and viability, TGFβ couples bone formation with bone resorption *(Janssens K et al, 2005; Fox SW & Lovibond AC, 2005)*.

1.7.1.2.2 Bone morphogenic proteins (BMP) and growth differentiation factors (GDF)

Bone morphogenic proteins (BMPs) and growth and differentiation factors (GDFs) form a single family of cystine-knot cytokines, sharing the characteristic tertiary structure of the transforming growth factor β (TGF-β) superfamily *(Rider CC & Mulloy B, 2010)*. Besides the ability to induce bone formation, which gave the BMPs their name, the BMP/GDFs display morphogenetic activities in the development of a wide range of tissues: BMP-7 for kidney *(Dudley AT et al, 1995; Luo G et al, 1995)*, BMP-9 for liver *(Chen C et al, 2003)*, BMP-10 for heart *(Chen H et al, 2004)* and GDF-9/BMP-15 for germ cells *(Nicholls PK et al, 2009)*.
BMP/GDF homo- and hetero dimers interact with combinations of transmembrane serine-threonine kinase receptor dimers (type I and type II) to produce multiple possible signalling complexes, leading to the activations of one of two competing sets of SMAD transcription factors. Phosphorylated Smad complexes translocate into the nucleus and activate the transcription of target genes.

BMP/GDFs have a highly specific and localized function. These are regulated in a number of ways: 1) through the developmental restriction of BMP/GDFs expression and 2) through the secretion of several specific BMP antagonist proteins that bind with high affinity to the cytokines.
Among the high-affinity BMP antagonists is follistatin.
Fine tuning of control of BMP activity resulting from the balanced expression of the BMPs and antagonist noggin which is not only important within the skeleton, but also in the morphogenesis of a number of organs *(Rider CC & Mulloy B, 2010)*.
Finally, other BMP antagonists are distant members of the TGF-β superfamily and are referred to as the CAN (Cerberus and Dan (differential screening-selected gene aberrative in neuroblastoma)) family of proteins.

As BMPs regulate cell differentiation and function as morphogens, they must act in a highly restricted, localized manner. Once released by proteolytic cleavage from their large membrane-bound precursor proteins, they are small, readily diffusible glycoproteins. The juxtacrine activity is achieved by two mechanisms. One is to restrict diffusion for a mature BMP protein by remaining associated to its larger pro-domain. This has been proven for BMP-7 : the pro-domain anchors BMP-7 within the extracellular matrix through binding to fibrillin-1 *(Gregory KE et al, 2005)*. Secondly and more widely established is the binding of the mature released cytokine to the highly acidic heparan sulphate (HS) glycosaminoglycan found on the cellsurface and in the extracellular matrix, or to its more experimentally amenable variant, heparin *(Rider CC & Mulloy B, 2010)*. BMP-2 and -4 both interact this way, and thereby functionally restrict their activity to their location *(Ruppert R et al, 1996; Ohkawara B et al, 2002)*.

1.7.1.3 Insuline-like growth factors (IGFs)

Insuline-like growth factors (IGFs) exerts positive effects on bone size, bone formation and bone density in mammals *(Kawai M et al, 2009)*. Increased bone formation and enhanced trabecular and cortical bone volumes were observed in mice overexpressing IGF-1 in osteoblasts *(Zhao G et al, 2000)*. Deleting IGF1 receptor in osteoblasts caused impaired bone formation and reduced mineralization *(Zhang M et al, 2002)*.
The two IGFs, IGF-1 and IGF-2, bind to and signal through the IFG1 receptor, a ligand-activated tyrosine protein kinase, which activates multiple intracellular signaling pathways in osteoblasts and other cell types *(Nakae J et al, 2001)*.
IGF binding protein 5 (IGFBP-5) inhibits IGF action in bone cells and blocks both osteobast differentiation and bone growth *(Mukherjee A & Rotwein P, 2008)*. Despite the multiplicity of signaling cascades activated by the IGF-1 receptor, growing evidence points to the phosphatidyl-inositol 3-kinase (PI3-kinase)-Akt pathway as being critical for IGF action in bone *(Giustina A et al, 2008; Mukherjee A & Rotwein P, 2009)*.

This pathway is required for BMP-2 mediated osteblast differentiation in culture *(Mukherjee A & Rotwein P, 2009)*.
The three Akt proteins found in mammals are structurally and functionally similar to serine-threonine protein kinases. Loss of Akt2 prevents the induction of Runx2 gene expression. Its potential to promote osteogenic development contrasts with its limited role in myogenesis from the same mesenchymal cell progenitors, in which Akt1 is critical for differentiation *(Rotwein P & Wilson EM, 2009)*.

IGF mediated signaling pathways differentially control mesenchymal cell fate and function. Signaling through the IGF-1 receptor also has been associated with enhanced aging in experimental animals *(Yang J et al, 2005; Rodriguez S et al, 2007)* and with increased cancer risk in humans *(Larsson O, 2007)*.

1.7.1.4 Cytokines and growth factors in bone metabolism

Cellular events involved in bone remodeling are modulated by a group of local factors. These local factors, or osteotropic cytokines, have extremely potent effects on bone cells.
Although it has long been recognized that inflammation, a consequence of immune-driven processes, significantly impacts bone turnover, the degree of centralization of skeletal and immune functions has begun to be dissected only recently *(Weitzmann MN, 2013).*

1.7.1.4.1 Receptor activator of NF- κB ligand (RANKL)

It is now recognized that formation of osteoclasts, the bone resorbing cells of the body, is centered on the key osteoclastogenic cytokine, receptor activator of NF-κB ligand (RANKL). Although numerous inflammatory cytokines are recognized to promote osteoclast formation and skeletal degradation, with just a few exceptions, RANKL is considered to be the final downstream effector cytokine that drives osteoclastogenesis and regulates osteoclastic bone resorption.

1.7.1.4.2 Interleukins (IL) and Tumor necrosis factor α (TNFα)

Cytokines and growth factors are important in bone tissue as mediators of cell-to-cell and matrix-to-cell communication. Cytokines locally mediate the effects of several hormones on bone cells. Indeed, calciotropic hormones (sex steroids, parathormon, growth hormone) modulate the bone-cell production rate of these factors and, conversely, cytokines can change the number of receptors for the hormones on bone cells.

Estradiol modulates osteoblastic cytokines acting on osteoclast differentiation. In mice, increased interleukin-6 production by osteoblasts is responsible for increased bone resorption occurring after ovariectomy *(de Vernejoul MC et al, 1993).*

Both osteoblasts and osteoclasts are derived from progenitors that reside in the bone marrow; osteoblasts belong to the mesenchymal lineage of the marrow stroma, and osteoclasts to the hematopoietic lineage. The development of osteoclasts from their progenitors is dependent on stromal-osteoblastic cells, which are a major source of cytokines that are critical in osteoclastogenesis, such as interleukin-6 (IL-6) and interleukin-11 (IL-11). The production of IL-6 by stromal osteoblastic cells, as well as the responsiveness of bone marrow cells to cytokines such as IL-6 and IL-11, is regulated by sex steroids *(Manolagas SC & Jilka RL, 1993)*. When gonadal function is lost, the formation of osteoclasts as well as osteoblasts increases in the marrow, both changes apparently mediated by an increase in the production of IL-6. Estrogens and androgens are protective on the skeleton. Both of these steroids inhibit the transcriptional activity of the IL-6 gene promoter via mechanisms involving their respective specific receptors *(Manolagas SC, 1995)*. Although IL-6 seems an essential pathogenic factor in the bone loss caused by gonadal deficiency, other cytokines IL-1, IL-11 and tumor necrosis factor α (TNFα) may also be involved in upregulation bone resorption. However, in contrast to IL-6, these cytokines do not seem to be directly regulated by sex steroids *(Manolagas SC, 1995)*.

The cellular activity of the bone marrow is also altered by the process of aging. Specifically, senescence may decrease the ability of the marrow to form osteoblast precursors. Like homeostasis of other regenerating tissues, homeostasis of bone depends on the orderly replenishment of its cellular constituents. The disruption of the balance between bone resorption and bon formation results in the loss of bone. Excessive osteoclastogenesis and inadequate osteoblastogenesis are responsible for the mismatch between the formation and resorption of bone in postmenopausal and age-related osteopenia. The recognition that changes in the numbers of bone cells and the changes in the activity of individual cells, form the pathogenetic basis of osteoporosis is a major advance in understanding the mechanism of this disease.

IL-1, TNFα and M-CSF that have long been associated with osteoclastic bone loss, act by promoting RANKL production by osteoblast precursors (bone marrow stromal cells (BMSC) and/or mature osteoblasts *(Hofbauwer LC et al, 1999 , Wei S et al, 2005)* and/or by reducing OPG production *(Wei S et al, 2005)* and/or by upregulating the receptor RANK on osteoclast precursors *(Arai F et al, 1999)*.

It was further demonstrated that IL-1 mediates the osteoclastogenic effect of TNFα by enhancing stromal cell expression of RANKL and directly stimulating differentiation of osteoclast precursors *(Hofbauwer LC et al, 1999).*

1.7.1.4.3 Transforming growth factor β (TGFβ)

Although TGFβ has been shown to stimulate early bone formation, several studies have shown a capacity of this cytokine to also promote vitamin D-dependent production of osteoclasts in osteoblast cocultures *(Shinar DM & Rodan GA, 1990)* and to augment in vitro RANKL-mediated osteoclast formation *(Sells Galvin RJ et al, 1999).*

In contrast, TGFβ has also been reported to inhibit osteoclastogenesis *(Shinar DM & Rodan GA, 1990)* mainly, at high concentrations *(MN et al, 2000)*. Estrogen promotes apoptosis of murine osteoclasts through production of TGFβ *(Hughes DE et al, 1996)*. B cells in vitro are inhibitory to osteoclastogenesis as they secrete significant concentrations of TGFβ, leading to apoptosis of osteoclasts and their precursors *(Weitzmann MN et al, 2000)*.

Under conditions of estrogen deficiency in mice in vivo, it was further shown that a decline in TGFβ production is permissive for T-cell-driven inflammatory bone loss, as TGFβ is a potent inhibitor of T-cell activation. Consequently, estrogen-induced TGFβ may act to protect the skeleton from the ravages of inflammation *(Gao Y at al, 2004).*

Interestingly, one study has reported direct TGFβ-induced osteoclast formation by a RANKL-independent mechanism *(Itonaga I et al, 2004)*. While RANKL stimulates large osteoclasts, TGFβ induced large numbers of mononucleated preosteoclasts and small mature osteoclasts producing many small resorption lacunae *(Itonaga I et al, 2004)*.

The significance of TGFβ in the regulation of basal osteoclastogenesis in vivo remains unclear with the potential for RANKL-dependent and -independent effects as well as direct inhibitory effects *(Itonaga I et al, 2004)* through modulation of RANKL and OPG, and through suppression of T-cell activation, creating a complex interplay.

1.7.1.4.4 Tumor necrosis factor α (TNFα)

TNFα is a non-glycosylated protein of 157 amino acids with a pleiotropic pro-inflammatory cytokine activity *(Chen G & Goeddel DV, 2002; Bradley JR, 2008)*. TNFα is produced primarily by activated macrophages but also by a variety of other structural cell types, including fibroblasts, smooth muscle cells and osteoblasts *(Chen G & Goeddel DV, 2002; Bradley JR, 2008)*. Biological responses of TNFα are mediated by the specific binding to a type I or type II receptor expressed on the surface of many cells. The binding of TNFα to its receptors results in the activation of an inflammatory response classically mediated by a wide variety of pro-inflammatory cytokines, including interleukins, interferon-γ and chemokines *(Chen G & Goeddel DV, 2002; Bradley JR, 2008)*. In addition, intercellular signal transduction generated by TNFα elicits a wide spectrum of other cellular responses, including the modulation of the proliferation and

differentiation of a variety of cell types and the induction of apoptosis via several pathways *(Bradley JR, 2008)*, such as the meiosis-specific serine/threonine protein kinase (MEK) pathway, the extracellular signal-regulated kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK) pathway, the p38 kinase pathway and the NF-κB pathway.

Signal transduction of the TNFα pathway occurs partly through the activity of the NF-κB family of transcriptional factors. In unstimulated cells, NF-κB is predominantly localized in the cytoplasma as part of a complex of inhibitory IκB proteins. In response to a variety of stimuli, such as TNFα or IL-1β, IκBs are phosphorylated by the activated IκB kinase (IKK). IKKβ is most critical for the classical NF-κB pathway, that depends on IκB degradation. In this pathway, the p50/p65 heterodimer enters the nucleus and binds to NF-κB-responsive elements to regulate the expression of genes that are involved in the regulation of immune and inflammatory responses, proliferation, tumorigenesis, and survival *(Ghosh S & Hayden MS, 2008; Karin M et al, 2008)*.
In contrast to NF-κB, BMP signaling upmodulates proliferative and differentiation signaling in osteoblasts and other tissues *(ten Dijke P, 2006)*. The BMP/Smad and NFκ-B signaling systems thus exert antagonistic effects.

1.7.1.5 Integrins

To maintain the low pH requisite for its resorptive activities the osteoclast has a physical intimacy with the underlining bone. Integrins are transmembrane, heterodimeric proteins consisting of an α- and β-chain *(Ruoslahti E & Pierschbacher MD, 1987)*. The external domain recognizes extracellular matrix proteins (e.g. bone vitronectin, osteopontin and bone sialoprotein *(Ross FP et al, 1993)*) via interaction with specific amino acid motifs, i.e. for the bone proteins: Arg-Gly-Asp. The cytoplasmic domains of the heterodimers associate with intracellular molecules, involved in downstream signaling and organization of the cytoskeleton *(Ruoslahti E, 1996)*. Given their capacity to bind extracellular matrix proteins, integrins are likely candidates to mediate osteoclast-bone attachment. Studies demonstrated that $\alpha_v\beta_5$ is a marker for the osteoclast precursor and $\alpha_v\beta_3$ for the mature osteoclast *(Inoue M et al, 1998; McHugh KP et al, 2000)* and both are functionally important. A variety of proinflammatory cytokines involved in osteoclastogenesis modulate expression of $\alpha_v\beta_5$ and $\alpha_v\beta_3$.

Osteoclastic bone resorption is a multistep and cyclical process involving dramatic changes in the actin cytoskeleton. During the resorptive phase, the cell organizes its fibrillar actin in a ringlike structure surrounding a resorptive microenvironment (the clear zone). Accordingly the osteoclast contributes to the isolation of this acidic space from the general extracellular milieu *(Vaananen HK & Horton M, 1995)*.
The morphology of osteoclast generated from β3-/- marrow macrophages, more specifically their failure to spread in culture, suggests an abnormal actin cytoskeletal formation in the absence of $\alpha_v\beta_3$ *(Teitelbaum S, 2000a; McHugh KP et al, 2000)*.
In, knockout mice (β_3 -/-) bone histology demonstrates substantial increases in trabecular bone mass and cortical bone thickness. Deletion of the $\alpha_v\beta_3$ integrin results in osteoclast dysfunction, prompting osteosclerosis.

In additon to anchoring cells to extracellular matrix proteins, integrins transmit matrix-derived, intracellular signals. In the case of $\alpha_v\beta_3$, such signals appear to be mediated by the β_3 cytoplasmatic domain interacting with intracellular proteins. Failure to form a normal ruffled border by β_3 -/- osteoclasts indicates that intracellular signals transmited by this intergin are essential to achieve the complete osteoclast phenotype. The blokkade of $\alpha_v\beta_3$ has been proposed as a potential novel avenue for the prevention and treatment of osteoporosis.

1.7.1.6 Interaction of immune and bone cells

The regulatory effects of the immune system on the skeleton during homeostasis and activation of bone remodeling have been known for years. More recently it has also become evident that bone tissue is capable to regulate immune cell development.

In the bone marrow, the differentiation of hematopoietic progenitor cells requires specific microenvironments, called "niches", provided by various subsets of stromal cells many of which are of mesenchymal origin.
Within the osteoblast lineage, distinct differentiation stages exert differential regulatory effects on hematopoietic development. The critical role of osteblast progenitors has been reviewed *(Panaroni C & Wu JY, 2013)*. Further studies are needed to characterize different stromal populations involved in order to better understand their contribution to the generation of unique bone marrow (BM) niches, their roles in the cross-talk with hematopoietic cells and the temporal and spatial mechanisms by which they can regulate the cell cycle and differentiation programs of hematopoietic cells.

1.7.1.7 Adipokines: leptin, adiponectine

Leptin, a hormone firstly described in 1994, inhibits appetite and favors engery expenditure *(Ducy P et al, 2000b; Spiegelman BM & Flier JS, 2001; Auwerx J & Staels B, 1998)*. The protein is synthesized and secreted by adipocytes. So leptin could be a fat messenger *(Considine RV et al, 1996 ; Sinha MK et al, 1996, Queresh A et al, 1997, Flier JS, 1998)* or could have a role as regulator of fat mass *(Campfield DJ et al, 1995 ; Pelleymounter MA et al, 1995 ; Chua SC et al, 1996, Collins S et al, 1996 ; Van den Saffele JK et al, 1999)*.
Another function of leptin, described in rodents but not confirmed in humans, is the initiation and maintenance of gonadal function *(Ahima RS et al, 1997; Cheung C et al, 1997)*.
Leptin fulfills its cardinal functions after its binding to its single receptor localized on neurons in the brain *(Tartaglia LA et al, 1995 ; Ducy P et al, 2000b; Spiegelman BM & Flier JS, 2001)*.
In knockout mice, the absence of leptin or of its receptor in these hypogonadic animals led to a high bone mass phenotype affecting trabecular bone, as bone formation was stimulated *(Ducy P et al, 2000b)*.
Leptin is now widely accepted as an inhibitor of bone mass accrual *(Elefteriou F et al, 2004)*.
Leptin does, however, appear to affect trabecular bone differently than cortical bone. As originally described *(Ducy P et al, 2000b ; Takeda S et al, 2002)*, leptin deficient ob/ob mouse have a "high bone mass" phenotype, based on the analysis of trabecular bone density. However, this differential activity may not be the case in leptin deficiency in humans (e.g. patients with anorexia).
The effect of leptin is indirect and involves the central nervous system (CNS). Injection of leptin in the CNS reproduces the effect of circulating leptin. Signals originating from the CNS target the osteoblast and therefore control bone formation. The hypothalamus is a key region for the control of bone mass. Ventromedial hypothalamic neurons are the site of this control center. The sympatic nervous system is the mediator of the signals sent by hypothalamus. Leptin functions as a β-adrenergic agonist. In the absence of leptin, sympatic activity decreases and bone mass increases.

Osteoblasts express ß adrenergic receptors which are linked to two different signaling systems. One of them is the transcription factor AP-1 which promotes bone formation. This pathway determines the overall effect of leptin on bone. On the other hand, leptin stimulates the expression of CART (cocaine- and amphetamine-regulated transcript) in the hypothalamus *(Elefteriou F et al, 2005)*. CART decreases the expression of RANKL, thereby reducing bone resorption. The overall effect of these two actions of leptin is to inhibit bone resorption.

Although reports on leptin's effects on bone occasionally appear to be inconsistent or even contradictory, the bulk of literature on leptin and bone points to some basic conclusions *(Reid IR et al, 2006)*: (1) Leptin deficiency, following caloric restriction or due to a congenital absence, is associated with low total bone mass, due to decreased cortical bone formation; (2) Leptin resistance increases with age, estrogen deficiency and with increases in endogous leptin. Hence, the majority of postmenopausal women with normal food intake are relatively insensitive to leptin treatment or to variations in circulating leptin levels; (3) The problem of leptin resistance indicates that the potential use of leptin in bone loss is greatest in conditions of leptin deficiency and energy deprivation, such as food restriction, exercise-induced hypothalamic amenorhea, anorexia nervosa, and perhaps weight loss.

The bone is a target of hormones, such as leptin, which are important modulators of food intake and metabolism. In turn, osteoblasts release a hormone which appears to modulate glucose and fat metabolism **(Figure 27)**. Osteoblasts are the unique site of production for osteocalcin, a protein known to favor mineralization of the extracellular matrix. To do so, osteocalcin is first converted into its Gla derivative. This conversion is catalysed by osteocalcin protein tyrosine phosphatase (OST-PTP). It has

been proposed from animal studies that, in the absence of OST-PTP, Gla osteocalcin is no longer produced and osteocalcin accumulates. In this condition, osteocalcin acts as a hormone: it stimulates proliferation of pancreatic b-cells and increases insulin secretion as well as the sensitivity of peripheral tissues to insulin.

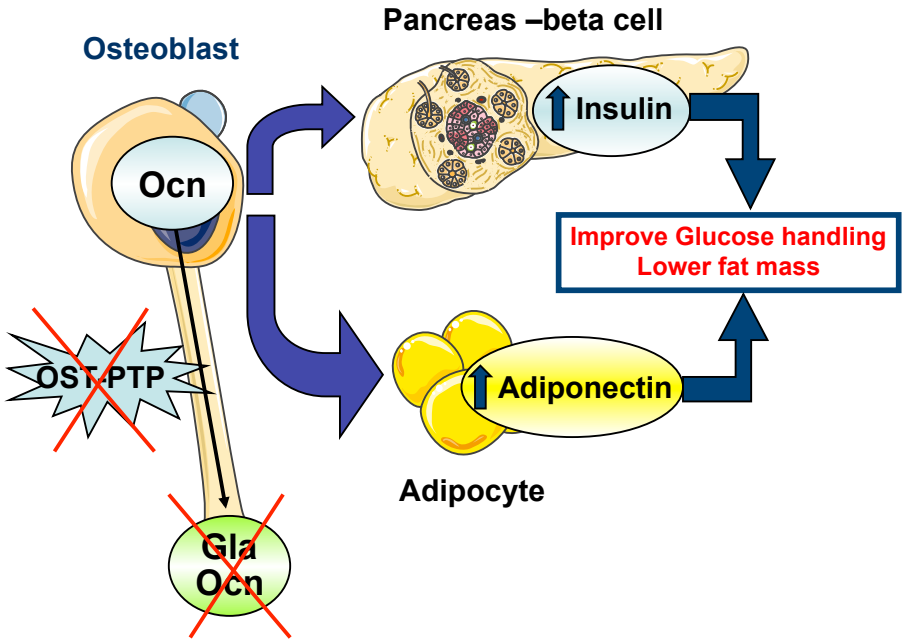


Figure 27: Hormonal effects of osteocalcin on pancreatic –cells and adipocytes (Reproduced from Friedlander G 2012).

Furthermore, osteocalcin stimulates adipocytes to produce adiponectin. Both insulin and adiponectin influence metabolism by improving glucose metabolism and reducing fat mass. Osteocalcin appears therefore as a hormone potentially able to oppose risk factors such as those encountered in type 2 diabetes.

1.7.1.8 Genetic factors

1.7.1.8.1 Single nucleotide polymorphisms

Twin and family studies have demonstrated that bone mineral density (BMD), one of the most commonly studied phenotypes, is highly heritable, as is bone geometry and bone ultrasound variables *(Karasik D, 2002; Karasik DE, 2001; Karasik D, 2007)*.
In a three-generation family study (FAMOS) on idiopathic osteoporosis (IO) in men, we provided arguments for an inherited deficient acquisition of bone during maturation *(Van Pottelbergh I, 2007)* **(Figure 28)**.

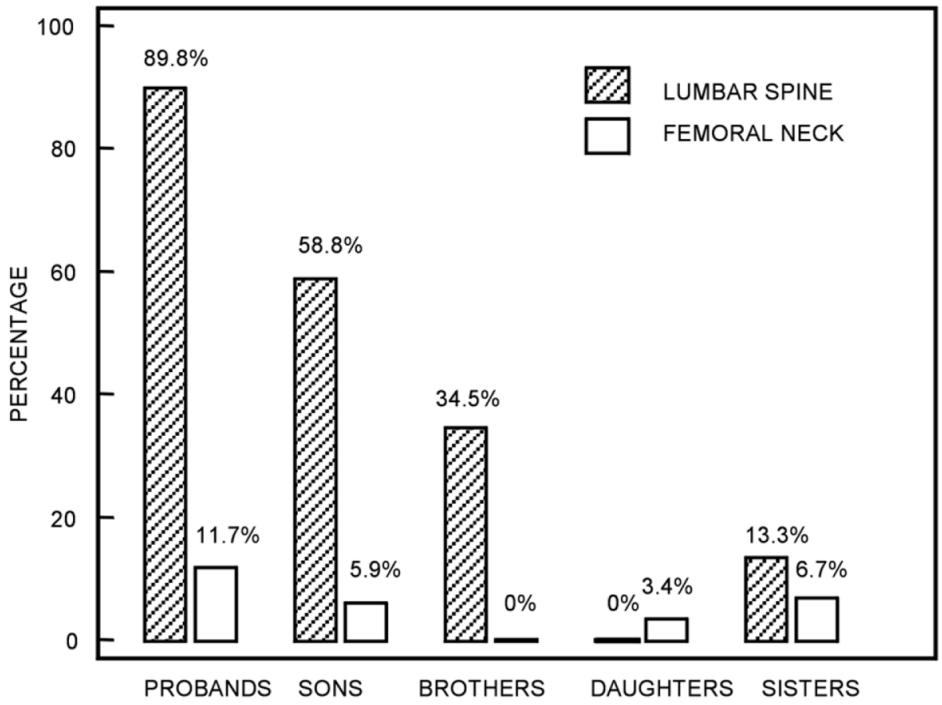


Figure 28: percentage of subjects with z-score <-2.0 at the lumbar spine and the femoral neck in 61 men with idiopathic osteoporosis and their male and female children and siblings (Reproduced from van Pottelbergh I, 2003).

Later we extended these observations on the skeletal phenotype in men and their first-degree relatives. The bone mass deficit observed in men with IO involves both trabecular and cortical bone and results from both lower vBMD and smaller cortical bone size. Since a similar bone phenotype is evident in their sons and there is no evidence for increased bone loss in our male study population with IO, this supports the view that IO results from deficient bone acquisition *(Lapauw BM et al, 2009b)*. In addition, we propose a mediating role for deficient estrogen action in the pathogenesis of male IO, supported by observations of lower serum FE_2 levels, lower vBMD, larger endosteal circumferences and lower sternum height in these men *(Lapauw BM et al, 2009b)*.

Significant heritability of fracture has also been demonstrated for hip fracture occuring at younger ages and for vertebral fractures *(Michaelsson K, 2005; Liu, CT, 2012)*. The search for genes underlying these skeletal phenotypes has matured over the past decade, beginning with linkage analyses in family cohorts *(Johnson ML, 1997; Styrkarsdottir U, 2003; Deng HW, 2002(a); Deng HW, 2002 (b))* and candidate gene association studies as well *(Iloannidis JP et al, 2004; Ralston SH et al, 2006; Langdahl BL et al, 2008; van Meurs JB et al, 2008)*.
Linkage analyses have not been successful in narrowing the number of candidate loci to specific genes responsible for transmition of the trait through families. Linkage analysis has relatively low power for complex disorders such as osteoporosis. Candidate gene studies, while used with some succes in identifying genes related to bone density and geometry, are limited to the study of a set of genes known to be related to bone biology.
Furthermore, the results of many of the early positive candidate gene studies were not confirmed in subsequent work.

These early successes and failures set the stage for a more non-hypothesis driven genome-wide search for osteoporosis genes with the advent of improvements in genotyping technology and with the development of computer storage platforms and statistical methods to analyze large amounts of data. Within the past five years, the field of skeletal genetics has moved into the era of genome-wide association (GWA) studies *(Altshuler D et al, 2008; Hindorff LA et al, 2009; Manolio TA, 2009 (a))*.

1.7.1.8.2 Genome wide association studies

Genome-wide association studies use high throughput genotyping of hundreds, thousands and even millions of the most common form of genetic variant, the single nucleotide polymorphism (SNP), and relate these SNP's to various phenotypes. This approach is powerful in that it interrogates the entire genome for associations between the variants and the phenotypes. The resulting stringent levels of statistical tests needed to achieve a "significant" association require a large sample size. To achieve large samples required to perform GWA studies, cohorts across the world have developed consortia required to undertake large meta-analyses of the GWA studies from individual cohorts.
To combine data across cohorts, studies typically "impute" millions of SNP's that are not actually genotyped on the platforms. Robust methods have emerged allowing for the imputation of genotypes based on work from the HapMap project *(de Bakker PI et al, 2008; Marchini J et al, 2007)*. All publications of GWA study results are recorded on a website maintained by the National Genome Research Institute studies (http://www.ebi.ac.uk/fgpt/gwas/).

The first GWA study for DXA-derived BMD and hip geometry traits was performed in the Framingham Study using a genotyping platform with only 100,000 SNP's in a sample of only 1,141 men and women. This study yielded no genome-wide significant findings *(Kiel DP et al, 2007)*.
The largest meta-analyses to date have been conducted by the Genetic Factors for Osteoporos (GEFOS) consortium. In the first meta-analysis from the GEFOS consortium *(Rivadeneira F et al, 2009)*, using DXA-derived BMD phenotypes of the spine and hip, 20 loci reached genome-wide significance (GWA; $P<5x10^{-8}$), of which 13 mapped to regions not previously associated with BMD. The other seven loci mapped to genes known to be involved in skeletal metabolism such as 1p36 (ZBTB40), 6q25 (ESR1), 8q24 (TNFRSF11B, osteoprotegerin), 11q13.4 (LRP5), 12q13 (SP7, osterix), 13q14 (TNFSF11, RANKL), and 18q21 (TNFRSF11B, RANK).

In the second meta-analysis from the GEFOS consortium *(Estrada K et al, 2012)* using DXA-derived BMD of the hip and spine from 17 GWA studies, the top-associated SNP's were tested for replication. These independent samples for replication came from a previously funded consortium called Genetic Marker for Osteoporosis (GENOMOS). This meta-analysis identified 56 loci (of which 32 novel) associated with BMD at the genome-wide significant level ($p< 5x10^{-8}$). These novel findings, as well as the confirmation of previous findings allowed to conclude that the implicated genes clustered within the RANK-RANKL-OPG, mesenchymal-stem-cell differentiation, endochondral ossification, and the Wnt signaling pathways.

In collaboration with the INSERM Unit 606 in Paris and INSERM Unit 563 in Toulouse we performed a GWAS analysis in pedegrees of men with idiopathic low bone mass *(Kaufman JM et al, 2008)*. Four of quantitative trait loci (QTL) reached the genome-wide criteria for significant or suggestive linkage. Apart from 22q11, which is a novel QTL, all other loci provide consistent replication for previously reported QTLs for BMD and other bone-related traits. Several of our specific-linkage areas encompass prominent candidate genes: type1collagen (*COL1A1*) and the sclerosteosis/van Buchem disease (*SOST*) genes on 17q21–23; the low-density lipoprotein receptor-related protein 5 (*LRP5*) gene on 11q12–13; and the *RANKL* gene on13q12–14.

In addition, the meta-analysis discovered loci containing genes not known to be involved in bone biology. It was found that 14 BMD loci were also associated with fracture risk. There remains a considerable unexplained proportion of the heritability observed for BMD, as is true of other complex phenotypes *(Manolio TA, 2009b)*. The unexplained heritability of bone phenotypes using GWA studies suggests that there may be larger numbers of variants with small effects, or rare variants with larger effects. Also, the possibility exist that there are structural variants, such as copy number variants, that have not been captured on some the genotyping arrays. There may also be gene by gene interactions that the current studies have not been powered to detect.
The next steps will involve a more thorough testing of the indentified loci for discovery of the functional variants, finer mapping and sequencing of promising loci, and studies of gene and environment interactions.

1.7.1.8.3 Epigenetics factors

Epigenetics are the mechanisms to modulate gene expression not depending on the underlying DNA sequence. These mechanisms include chemical modification of DNA and histone proteins. Understanding of the epigenetic modification or "epigenome" in the human genome is rapidly growing *(Kubota T & Hata K, 2013)*. Newly identified molecules related to DNA methylation have expanded our understanding of various congenital diseases *(Hata K et al, 2002)*. Molecules related to histone modification and advances in next-generation sequencing (NGS) technology led to the conclusion that many congenital diseases in which the underlying genetic cause was not identified are caused by mutations in the genes hat encode histone modification enzymes *(Kleefstra T et al, 2012)*. In disorders of low or high bone mass (e.g. osteogenesis imperfecta (OI), Ehlers-Danlos syndrom (EDS) and osteopetrosis (OPT) or skeletal dysplasias), next-generation sequencing panels, allowing simultaneous sequencing of different genes, provided a fast and accurate methods to arrive at a molecular diagnosis in most of these patients *(Sule G et al, 2013)*.

1.7.1.8.4 Telomeres, telomere length, telomerase and aging

Telomeres, the termini of linear chromosomes, exert a key role in cellular aging. They consist of large but variable numbers of DNA oligomer repeats embedded in a nucleoprotein complex. These structures keep track of the cumulative replicative history of normal somatic cells and are postulated to play a role in clinical age-associated phenotypes and disorders at an older age *(Klapper W et al, 2001)*. In humans the telomere DNA sequence consists of tandem repeats of a noncoding TTAGGG hexamer *(Blackburn EH, 2000)*. The telomere length (TL) is largely (in humans for 40 to 80%) genetically defined *(Slagboom PE et al, 1994; Vasa-Nicotera M et al, 2005; Andrew T et al, 2006; Njajou OT et al, 2007)*, but also determined by an age dependent attrition. Progressive telomere shortening in implicated in senescence in vitro and ample evidence exist to support the hypothesis that TL is correlated with chronological age and aging phenotypes in vivo. TL has therefore been put forward as a marker for biological aging and was also reported to be associated with aging diseases.
Although equal at birth *(Okuda K et al, 2002)*, adult men generally have shorter TL than their female counterparts *(Benetos A et al, 2001)*, as a result of their higher telomere attrition rate *(Nawrot TS et al, 2004; Unryn BM et al, 2005; Bekaert S et al, 2007)*. One hypothesis links the gender differences in TL with the gender difference in body size and the gender gap in life expectancy. Men are generally taller than women and more cell devisions are required to obtain and maintain body size. This results in a shorter TL in men, providing a possible explanation for their lower live expectancy *(Stindl R, 2004; Aviv A et al, 2005)*.

Functional telomeres play an important role in the perservation of chromosamal integrity in mammals *(de Lange T, 2002)*. In normal somatic cells, the progressive telomere attrition leads to critically short telomers and replicative senescence, a state characterized by the absence and by replication of biochemical changes *(Allsopp RC et al, 1992)*. However, the telomere attrition is not only determined by the division rate of proliferating cells. Repair of single strand breaks associated with oxidative stress is less efficient in telomeres than in centromeric DNA, resulting in an incomplete telomeric DNA replication and an accelerated telomeric attrition *(von Zglinicki T et al, 1995; Petersen S et al, 1998; Sitte N et al, 1998)*. Thus, telomere attrition is further modulated by both the levels of oxidative stress and the cellular anti-oxidant capacity *(Serra V et al, 2003; Saretzki G et al, 2003)*.
At the level of the organism, the impact of TL on the complex aging process, whether of not reciprocal, is primarily assessed through cross-sectional epidemiological studies. In most cases peripheral blood leucocytes (PBL) are used as systemic TL measure, supported by the observation that TL is to a large extent conserved among different tissues *(Friedrich U et al, 2000; Okuda K et al, 2002)*.

Cells can be rescued from critical telomere shortening, and the natural machinery to avoid senescence and gain virtually eternal life is utilized in normal germ and stem cells, and abused in tumors: activation of telomerase *(Greider CW & Blackburn EH, 1985; Greider CW et al, 1987; Bodnar AG et al, 1998; Shay JW et al, 2005)*. This telomere elongating enzyme counteracts telomere attrition, but is not or insufficiently expressed in normal somatic cells, resulting in a net TL loss in these cells *(Shay JW et al, 2005)*. In vitro studies of cultered osteoblasts have found intriguing links between observed telomere shortening and proliferation. Reconstitution of these osteoblasts and their precursors with ectopic telomerase activity extended their lifespan without loss of bone formation potential in the latter *(Kveiborg M et al, 1999; Yudoh K et al, 2000; Yudoh K et al, 2001)*.

1.7.1.9 Mechanical factors

Bone continually adapts its (micro)anatomy to meet changing physical and biochemical demands. Osteblasts, osteclasts and osteocytes cooperate to integrate these physical and biochemical cues to maintain bone homeostasis.

Osteocytes coordinate the adaptation of bone to changing physical demands on the skeleton *(Bonewald LF, 2011)*. Upon sensing mechanical loads through their canalicular processes, osteocytes initiate a series of biochemical signaling events that coordinate the activity of osteoblasts and osteoclasts to increase bone mass *(Duncan R & Turner C, 1995)*. In this way, physical stimuli employ established biochemical pathways long known to participate in the maintenance of bone homeostasis. These pathways include parathyroid hormone (PTH) *(Salvesen H et al, 1994)*; insuline-like growth factor 1 (IGF-1) *(Reijnders CMA et al, 2007)* and prostaglandin signaling (PGE_2) *(Price JS et al, 2011)*. More recently, sclerostin has been discovered as an osteocyte protein responsive to mechanical loads and antagonizing bone formation *(Robling AG et al, 2007)* (see also paragraph 1.6.4.3.4.).

Sclerostin plays a central role in the anabolic response of bone to mechanical loading. Applied loads repress sclerosting mRNA and protein expression *(Robling AG et al, 2007)*, thereby releasing the brakes on new bone synthesis. Several pathways that control bone development and metabolism also regulate the sclerostin-encoding gene, SOST. The BMP pathway induces SOST expression during bone development *(Kamiya N et al, 2008)*. PTH, an essential regulator of mineral homestasis, represses SOST expression *(Keller H et al, 2005)*. The rapid increase of PGE_2 following mechanical load also contributes to the mechanosensitive repression of SOST, although the mechanism remains to be identified *(Kitase Y et al, 2010)*.
The master osteoblast transcription factor Runx2 binds to and induces transcription of a more proximal element of the SOST promotor *(Sevetson B et al, 2004)*.

In combined investigations on genetically modified mouse models and in in-vitro approaches the effect of mechanical loads on TGFβ activity, the role of TGFβ in load-induced bone formation, and the regulatory relationships between TGFβ and sclerostin were evaluated *(Nguyen J et al, 2013)*. It was found that mechanical load rapidly represses the net activity of the TGFβ pathway in osteocytes, resulting in a reduced activity of downstream effectors: Smad2 and Smad3. Loss of TGFβ sensitivity compromises the anabolic response of bone to mechanical load, demonstrating that the mechanosensitive regulation of TGFβ signaling is essential for load-induced bone formation. Sensitivity to TGFβ is required for the mechanosensitive regulation of sclerostin, which is induced by TGFβ in a Smad3- dependent manner. These results show that physical cues maintain bone homestatis through the TGFβ pathway with the regulation of sclerostin expression and the deposition of new bone.
Although many pathways modulate the mechanical impact on bone metabolism, these pathways do not fully explain the complexity of the mechanotransduction in bone and the regulation of SOST expression.

Exercise and bone in rodents and humans shows mainly beneficial effects. However, a closer look reveals discordant results particularly depending on the age of practice **(Figure 29)**. A number of published studies indicate that the most effective period during which one observe a larger increase of bone mineral mass with exercise is during adolescence, especially during the pre-pubertal years (Borer KT, 2005; Kannus P, 1995).

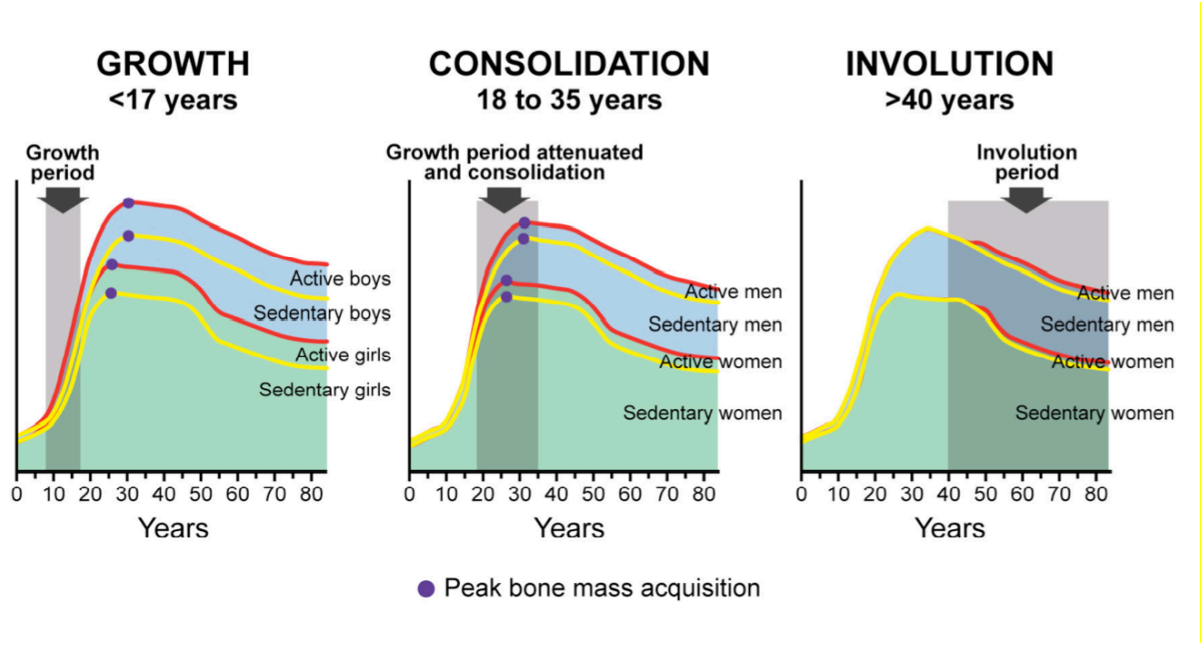


Figure 29 : Schematic description of the effect of physical activity on bone mass throughout life. The red curve represents a continuous exercise effect; otherwise a punctuated effect of exercise could be attenuated over time. The yellow curve represents a sedentary person engaging in normal activities (such as walking). Adapted from Blanchet C et al, 2000.

Physical activity should include activities that generate high ground-reaction forces, such as jumping, skipping and running and should last at least 7 to 20 months, to increase bone mass by about 2% at weight-bearing sites (femoral neck, spine) *(Rizzoli et al, 2010)*. Greater spinal BMC and areal BMD (aBMD), as well as trabecular volumetric density and bone strength in the peripheral skeleton, are also observed in adult elite gymnasts *(Eser P et al, 2009)*, suggesting that the effects of exercise on bone mass during growth are retained in adults. However, it has been reported that more than half of the BMC gain (+3.5% after 7 months of exercise versus controls) induced by jumping in pre-pubertal boys and girls (Gunter K et al, 2008) was lost over 8 years, that is even before these subjects reached peak bone mass. An initially greater bone mass (just after the intervention) may subsequently be remodeled to ultimately reach its genetically determined target.

Nutrition may influence the effect of exercise in children and adults. Calcium supplements in prepubertal girls have been shown to interact with their level of physical activity on bone mass gain. Moreover, exercise had no effect in children with a low calcium intake (453 mg/day), a mild effect in those with moderate intake (763 mg/day) and a greater effect in those with a high calcium intake (1073 mg/day) *(Rowlands AV et al, 2004)*. Similar observations have been reported for effects of protein intake and exercise on BMD and microarchitecture in young boys *(Chevalley T er al, 2008)*, which could be explained by a dual stimulation of insulin-like growth factor (IGF)-1 production by proteins and exercise.

The effects of exercise in adults are less prominent. In a meta-analysis pooling 14 studies, exercise resulted in a slightly higher BMD at the spine (weighted mean difference 0.016 g/cm^2, CI, 0.005 to 0.027, p=0.005) (Martyn-St James M, Carrol S, 2010). In older populations there are no published, randomized, prospective studies demonstrating positive effects of exercise on BMD. The lack of clear exercise effects in these populations may have several explanations. First is the difficulty in the elderly of practicing exercise with an impact of sufficient intensity to have a bone-forming effect. Moreover, a lack of motivation and fatigue limit compliance and the effectiveness of conventional exercise training. Second, lower muscle mass and strength (sarcopenia) in the elderly limits the strain exerted on the skeleton. Finally, the number of osteocytes and/or their sensitivity to mechanical strain may decline with age. The osteogenic potential of mesenchymal stem cells also declines with age. Nevertheless, despite no positive effect on BMD, several studies have demonstrated a reduction of fracture rate in elderly people practicing an activity, probably by preventing falls *(Korpelainen R et al, 2006; Karlsson MK, 2008; Sherrington C, 2008)*.

Regular physical exercise has been recommended as a low-cost and safe non-pharmacological strategy to counter the loss of bone mass that accompanies ageing. It has long been established that to improve bone density, bone tissue must be subjected to mechanical loading above that experienced in daily activities *(Frost HM, 1987)*. Mechanical loading should be dynamic, novel and involve high strain magnitudes and rates resulting in substantial overload *(Frost HM, 1987; Rubin CT, Lanyon LE (1984)*. Exercise trials indicate that, in women and men, the combination of high-impact loading exercises and moderate to high intensity resistance training is the most beneficial to prevent age-related bone loss *(Gomez-Cabello A et al, 2012).*

Effects of exercise varied greatly among studies. It appears that resistance training alone or in combination with impactloading activities are most osteogenic, whereas the walking trials had limited effect on BMD. Therefore, regular resistance training and impact-loading activities should be considered as a strategy to prevent osteoporosis in middle-aged and older peolpe. High quality randomised controlled trials are needed to establish the optimal exercise prescription.

1.8 Measurements of bone metabolism

1.8.1 Biochemical parameters

Bone turnover markers were already extensively discussed in paragraph 1.6.2.
The biochemical evaluation to diagnose secondary causes of disturbed bone metabolism are discussed in the second section of chapter 1 *(Kaufman JM & Goemaere S, 2008)*.

1.8.2 Evaluation of the mineral status

1.8.2.1 Dual X-ray absorptiometry (DXA)

Extensive epidemiologic data indicates that despite its limitations, the current gold standard for predicting fracture risk is an areal bone mineral density (aBMD) by Dual X-ray absorptiometry (DXA) *(Bilezikian JP et al, 2002; Bouxsein ML, 2003)*.
The first DXA scanners were introduced in the late 1980s, and DXA is now the most widely used and available bone densitometry technique *(Blake GM & Fogelman I, 2007)*. Dual X-ray energy beams are required to correct BMD measurements for overlying soft tissue and are produced by a variety of techniques by different manufacturers. The energies are selected to optimized separation of mineralized and soft tissues of the skeletal site analyzed.
Original DXA scanners used a pencil X-ray beam with a single detector, scanning in a rectilinear fashion across the anatomical site (scan times 5-10 min; spatial resolution 1mm). Technical developments introduced fan-beam X-ray sources and a bank of detectors to enable faster scanning (1min/site) with improved image quality and spatial resolution (0.5 mm) *(Fogelman I & Blake GM, 2005)*.

DXA can be applied to sites of the skeleton at which osteoporotic fractures occur. In the central skeleton this includes the lumbar spine and the proximal femur. DXA can also be applied to peripheral skeletal sites: e.g. the forearm and the calcaneum, using either full-sized or dedicated peripheral scanners. Central DXA measures of lumbar spine, femoral neck and total hip are currently used as the “gold standard” for the clinical diagnosis of osteoporosis by bone densitometry.

DXA X-ray attenuation values are converted to bone mineral content (BMC; g), bone area (BA; cm^2) is calculated by summing up pixels within the bone edges detected by the software algorithms. “Areal” bone mineral density (aBMD; g/cm^2) is calculated by dividing BMC/BA *(Engelke K & Gluer CC, 2006)*. Because the DXA image is a 2D image of a 3D object, the depth of bone is not taken into account. This fact results in DXA being size-dependent, one of its significant limitations, particularly in children in whom the bones

change in size and shape during growth, and in patients whose condition or disease might result in small stature or slender bones.

DXA provides aBMD of integral, i.e. cortical and trabecular bone. The cortical/trabecular bone ratios vary in different sites: 50/50 for the posteo-anterior lumbar spine; 10/90 for the lateral lumbar spine; 60/40 for the total hip; 80/20 for the total body; 5/95 for the calcaneus; 95/5 for the distal radius; 40/60 for the ultradistal radius.
Because of the different composition of bones and rate of change in various skeletal sites, measurements in different sites in the same individual will not give the same BMD results. Correlations between BMD measurements made in the same patient vary between r=0.4 and r=0.9 *(Grampp S et al, 1997)*.

The precision measures the reproducibility of the bone densitometry technique and is usually expressed as a coefficient of variation (CV; %). Precision of DXA varies by site from 1 to 2.5 %).
Accuracy is how close the BMD measured by densitometry is to the actual calcium content of the bone (ash weight). The accuracy of DXA lies in the region of 10-15%. The inaccuracies are related to marrow fat and DXA taking soft tissue as a reference *(Blake GM & Fogelman I, 2008)*.
With appropriate software, whole body DXA scanning can be performed, from which whole body BMC, regional BMC and body composition (lean, muscle and fat mass) *(Adams JE, 2008)* are extracted.
DXA thus makes some assumptions about body composition and soft tissues. So, inaccuracies may occur with excessively under- or overweigth subjects and with large changes in weight between scans. It is not known how this can be corrected for in adults.
Sensitivity is the ability of the measurement to discriminate between patients with and without fractures and to measure small changes with time and/or treatment. A statistically significant change in BMD is calculated as 2.77 multiplied by the precision (CV) at the site of measurement to provide the least significant change (LSC) *(Gluer CC et al, 1999)*. LSC for the spine, total hip and femoral neck are respectively 5.3%, 5.0% and 6.9%. Because changes in BMD are generally small, it is essential in an individual patient to leave an adequate time interval between DXA measures, usually 18-24 months.

Fracture prediction is the ultimate goal of the DXA measurement. Whether a patient develops a fracture depends on a number of factors in addition to BMD as assessed by DXA: age, a fall, the nature of the fall and the patient's response to the fall. It is impossible for BMD techniques to completely discriminate between those with and without fractures.

The lower the BMD, the higher the fracture risk of the patient *(Marshall D et al, 1996)*. BMD measurements made in any skeletal site (central and peripheral) are predictive of fractures. The relative risk (RR) of fracture per 1 SD decrease in BMD below the age-adjusted mean varies between 1.4 to 2.6 *(Marshall D et al, 1996)*. Site –specific measurements are best in predicting fracture in that particular anatomical place.

DXA involves very low radiation doses (calcaneus, 0.03μSv, forearm, 0.5 μSv; spine, 2-4 μSv; femur, 2-5 μSv; whole body, 1-3 μSv). These radiation doses are similar to those of the natural background radiation (2400 μSv/year or 7 μSv/day) *(Kalender WA, 1992)*.

There has been much debate on the appropriate use of bone densitometry. A population wide screening in postmenopausal women has not been established as cost-efficient program. However, there is consensus that central DXA is the "golden standard" measurement to assess fracture risk and make the diagnosis of osteoporosis in terms of bone densitometry *(Blake GM & Fogelman I, 2007; Compston J, 2005)*. Selection of patients who would most appropriately be referred for DXA is based on a case-finding strategy in those who have strong risk factors. There are national differences in such referral guidelines based on local reimbursement policies and economic constraints.
For the clinical interpretation of the BMD measurement, it is essential that after the exclusion of a variety of possible artefacts, proper age- and sex-matched reference data are used. A patient's result can be interpreted in terms of the standard deviations (SD) from the mean of either sex-matched peak bone mass (T-score) or age-matched BMD (Z-score). The World Health Organization (WHO) has defined osteoporosis in terms of bone densitometry as a T-score at or below -2.5 *(World Health Organization Study Group, 1994)*. This definition applies to DXA of lumbar spine, proximal femur (femoral neck) and distal third of the forearm. The definition does not apply for other techniques, e.g. QCT, QUS, or other anatomical sites, e.g. the calcaneus *(Miller P, 2000)* Nor it is yet confirmed to be applicable to younger women and men. In children and young adults up to 20 years of age, interpretation can be made only in comparision with age-matched mean.

1.8.2.1.1 Limitations of DXA

Epidemiologic studies and clinical trials have identified limitations of areal BMD measurement, including the following observations: (1) many fractures occur among patients with "normal" BMD *(Wainwright S et al, 2001; Miller PD, 2002)*; (2) small changes in areal BMD following antiresorptive therapy result in greater than expected reduction in fracture risk *(Cranney A et al, 2002a,b,c* and (3) changes in BMD following antiresorptive therapy explain a small proportion of the variance of the fracture risk reduction *(Cummings SR et al, 2002; Sarkar S et al, 2002; Delmas P & Seeman E, 2004; Watts NB et al, 2005)*.
Newer techniques have and are being developed and validated to assess various components of bone strength in order to improve the prediction of fracture and the monitoring of treatment response. The ability of bone to resist fracture under a given loading condition is determined by the bone's morphology, i.e. its size, shape and microarchitecture, as well as properties of the bone matrix *(Bouxsein ML, 2003)*. Imaging techniques that can measure one or more of these features may enhance clinical management of individuals with osteoporosis. Presently, most advanced noninvasive imaging tools are capable of measuring the amount of bone and how bone is arranged in space. However, few of these tools are able to access the intrinsic properties of the bone matrix itself.

1.8.2.1.2 Use of DXA to assess vertebral fracture

Lateral views of thoracic and lumbar spine (T4-L4) can be obtained with fan-beam scanners, using dual- or single-energy scanning **(Figure 30).**

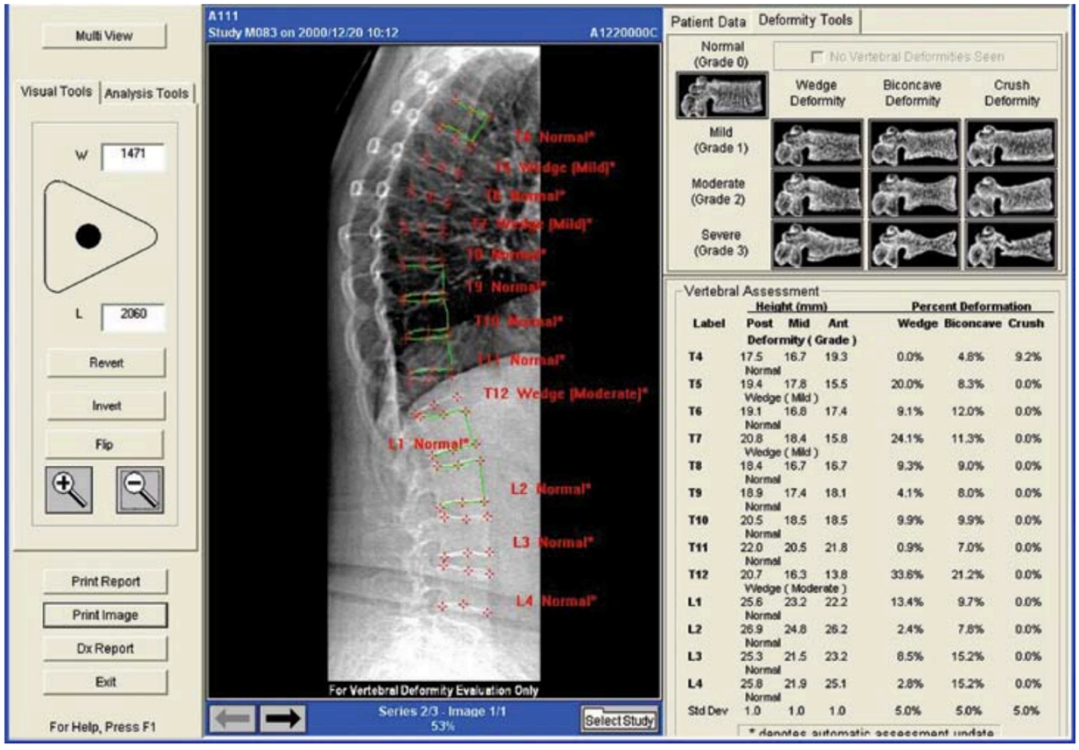


Figure 30: *Image obtained by QDR-4500A densitometer (Hologic, Inc., Bedford, MA, USA) with good visualization of vertebrae from T4 to L4. Vertebral morphometry shows two vertebral fractures as mild wedging to T5 and moderate wedging at T12.*

From these scans, visual assessments for vertebral features or mophometric assessments of vertebral shape can be made *(Ferrar L et al, 2005; Roberts MG et al, 2007)*. Vertebral fracture assessement (VFA) has several advantages over conventional radiography : lower dose of ionizing radiation and avoiding

problems of the divergent X-ray beam. The divergent beam of a conventional radiograph distords vertebral shape, causing apparent biconcavity of endplates, as a parallax effect on radiographs. Guidelines suggest that older patients who have low BMD with historical height loss of 4 cm in women and 3 cm in men, in combination with other risk factors should undergo vertebral fracture assessment using VFA on DXA devices *(Position statement of the International Society for Clinical Densitometry, 2005)*. The VFA methods has been shown to be statisfactory to exclude the presence of vertebral fractures *(Rea JA et al, 2000)*.

1.8.2.1.3 Use of DXA to assess bone structure

Based on initial studies of Martin and Burr (Martin RB & Burr DB, 1989), Thomas Beck has developed a technique, "hip structure analysis (HSA) ", that uses two-dimentional DXA scans to derive measurements of bone geometry at the proximal femur. Profiles of bone mass were derived from one bone margin to the other. Based on a number of assumptions of geometry and distribution of trabecular and cortical bone, several morphological parameters (e.g. bone area and width, cortical thickness and calculated bone strenght indices) could be derived. An extensive description of this method is provided in the "material and method" section of the manuscript about the femoral neck geometry in chapter 5.2. The HSA method is not a three-dimensional measurement, but is based on two-dimensional DXA technology and relies on several assumptions on shape and trabecular distribution. The inherent limitations are discussed in chapter 5.2
The HSA method has provided novel information regarding age-, race- and sex-related differences in femoral bone geometry that may contribute to the hip fracture risk *(Crabtree N et al, 2000; Nelson DA et al, 2000; Beck TJ et al, 2001 (a); Beck TJ et al, 2001 (b); Looker AC et al, 2001; Crabtree NJ et al, 2002; Beck T, 2003; Cauley JA et al, 2005)*. More recently, studies using HSA demonstrated effects of anti-catabolic and anabolic therapies on femoral bone geometry *(Greenspan SL et al, 2005; Uusi-Rasi K et al, 2005)*.

Presently, it is not clear whether HSA improves fracture prediction independly of BMD as recent data suggested that HSA-derived femoral bone geometry measurements did not predict fracture risk after adjusting for BMD *(Melton LJ et al, 2005)*. A number of assumptions of which the validity have not been established are implicit in the HSA method. Moreover, the method is inherently limited to a single plane, and therefore cannot fully reflect bone strength.
Alternative methodologies capable of directly measuring the three-dimensional geometry such as magnetic resonance imaging (MRI) and computed tomography (CT) are employed in clinical studies to better establish the relationship between bone geometry, bone density and fracture risk.

1.8.2.1.4 Use of DXA to assess microachitecture

The trabecular bone score (TBS) is a gray-level textural metric that can be extracted from the 2-dimensional lumbar spine dual-energy X-ray absorptiometry (DXA) image. TBS is related to bone microarchitecture and provides skeletal information that is not captured from the standard bone mineral density (BMD) measurement.
Based on experimental variograms of the projected DXA image, TBS has the potential to discern differences between DXA scans that show similar BMD measurements. An elevated TBS value correlates with better skeletal microstructure. A low TBS value correlates with weaker skeletal microstructure.
Lumbar spine TBS has been evaluated in cross-sectional and longitudinal studies *(Silva BC et al, 2014)*. First cross-sectionally *(Boutroy S et al, 2013; Del Rio LM et al, 2013)*, TBS gives lower values in post-menopausal women and in men with previous fragility fractures than their non-fractured counterparts. The TBS results are also lower in women who have sustained a fragility fracture but in whom DXA does not indicate osteoporosis or even osteopenia *(Hans D et al, 2011)*. It is consequently proposed that TBS is complementary to data available by lumbar spine DXA measurements *(Hans D et al, 2011)*. Secondly in longitudinal studies, TBS predicts fracture risk as well as lumbar spine BMD measurements in postmenopausal women. TBS is associated with fracture risk in individuals with conditions related to reduced bone mass or bone quality.
Finally, efficacious therapies for osteoporosis differ in the extent to which they influence the TBS *(Rizolli R et al, 2012)*. Based on the actual available data, lumbar spine TBS holds promise as an emerging technology that could well become a valuable clinical tool in the diagnosis of osteoporosis and in fracture risk assessment.

1.8.2.2 Quantitative Ultrasound of bone

Quantitative ultrasound (QUS) measurements were initially proposed as a technique capable of measuring bone "quality". Yet transmission ultrasound methods primarily reflect BMD *(Brandenburger GH, 1993)*. Several transmission ultrasound modalities have shown in prospective studies to predict fracture risk as well as BMD measurement *(Hans D et al, 1996; Bauer D et al, 1997)*. In the OPUS study *(Gluer CC et al, 2004)* some of the 5 compared techniques were as capable as DXA-BMD to discriminate vertebral fracture patients from non-fracture patients. Newer ultrasound imaging techniques applying acoustic impedance, backscatter and combined reflection and transmission measurements showed promise for examination of trabecular structure and material properties *(Chaffai S et al, 2002; Wear KA et al, 2001; Wear KA, 2003)*.

1.8.2.3 Quantitative computed tomography (QCT)

1.8.2.3.1 QCT to assess bone density and macroarchitecture

Quantitative computed tomography (QCT) scans of the proximal femur and spine are currently used to assess geometry and volumetric density of the trabecular and cortical bone compartments *(Lang TF et al, 2002; Lenchik L et al, 2004)*. This approach employs standard clinical CT scanners in combination with a bone mineral phantom used to calibrate the image data, providing a measurement of volumetric bone mineral density (vBMD) independent of bone size. This technique can by employed to evaluate the combined effects of changes in bone geometry and density with age and treatment *(Black DM et al, 2003; Rehman Q et al, 2002; Riggs BL et al, 2004)*. Standard resolution of QCT images is not adequate to assess trabecular bone microarchitecture, as they are limited to an in-plane resolution of 400 μm and slice thickness of 1 mm. More recently high-resolution imaging with multislice spiral CT scanners has achieved in-plane resolution of approximately 200 μm and slice thickness of 500 μm. This approach has been used in vivo to evaluate the lumbar spine, and appears to provide superior discrimination of fragility fracture patients compared to DXA BMD *(Ito M et al, 2005)*.
Peripheral QCT (pQCT) has mostly been carried out at the distal forearm. Using small-angle fan beams typical single slice settings achieve 1-3 mm slice thickness and an in plane pixel size of 100-300 μm. BMD and morphological parameters are measured at the distal radius (4% of radius length, mostly trabecular) and more proximal locations (14-66% of radius length, cortical bone).
The interest in QCT has two primary reasons : the deficiency of DXA in monitoring treatment and the prospect that QCT may not only perform better but also yield direct measures of strenght. However, prospective studies on risk assessment and clinical guidelines for appropriate use are lacking. The place of QCT and pQCT in clinical practice actually remains unclear and they remain mainly tools for research. Recent developments, however, document that the potential for further advances in QCT are very substantial.

1.8.2.3.2 QCT to assess trabecular bone microarchitecture

A high-resolution peripheral CT (HR-pQCT) system capable of achieving resolutions of up to 80 μm at tolerable radiation doses has been introduced. This technique is accurate for assessment of the trabecular architecture at peripheral sites, including the distal radius and distal tibia *(Boutroy S et al, 2005; Khosla S et al, 2005; Khosla S et al, 2006)*. Although BMD values were identical, HR-pQCT architecture measurement at the distal radius enabled to discriminate osteopenic women with a history of fragility fracture from those who had not fractured *(Boutroy S et al, 2005)*.
Microstructural assessment by high resolution CT (HRCT) allows visualization of trabecular microstructure and some aspects of cortical porosity. Best image quality at 80 μm voxel size can be achieved at periferal sites. Structural information can be extracted from the image *(Boutroy S et al, 2005; Sornay-Rendu E et al, 2007; McNeil JA & Boyd SK, 2007)*. It remains to be shown whether this improves vertebral and hip fracture prediction *(Melton LJ III et al, 2007)*.

HRCT of the spine (voxel size 160 to 200 μm) is sufficient to depict and quantify trabecular separation. An improved discrimination of the vertebral fracture population discrimination *(Ito M et al, 2005)* and responsiveness to treatment *(Graeff C et al, 2007)* has been demonstrated versus baseline.

1.8.2.4. Magnetic Resonance Imaging (MRI)

An alternative approach to assess trabecular microarchitecture employs clinical MRI scanners combined with special designed coils to generate images of trabecular architecture at peripheral sites. Using this approach, in vivo resolutions of 100-300 μm in plane and slice thickness of 250-500 μm have been achieved *(Link TM et al, 1999; Wehrli FW et al, 2002)*.
With this resolution, it is not possible to produce accurate values for most features of trabecular architecture. MR-based imaging of trabecular architecture appears promising but warrants additional investigation. The current limitation of high resolution MRI includes the relatively long acquisition time (10-15 min), requirement of specialized coils, and restriction to evaluate appendicular sites. Establisment of robust analysis methodologies and normative databases, have a potential for further clinical use.

1.8.2.5. Finite Element Analysis to estimate bone mechanical behavior

From the biomechanical viewpoint, an approach that accurately evaluates the three-dimensional geometry and heterogenous distribution of material properties of bone may provide improved estimates of bone strength. In this regard, finite element analysis (FEA) is of interest to assess bone biomechanical behavior. FEA has been widely used in engineering. In solid and structural mechanics, it is the computational modeling tool of choice, as it can provide an accurate estimation of how an object with a complex geometrical shape, e.g. a whole bone or trabecular network behaves, when it is subjected to external loads. The FEA approach to solid and structural mechanics problems begins by representing the object as a collection of a finite number of building blocks, or elements, each of which is defined by a small number of reference points, or nodes. The deformation of each element that occurs in response to the applied load is represented by simple yet versatile functions, known as shape functions, in which the only unknowns are the displacements of the nodes. Once the nodal displacements are computed, the strain distribution throughout each element, and hence the entire object, can easily be obtained.
To compute these displacements, the investigator must specify two types of information: first the applied loads on the structure under investigation and second the material properties for each element. Thus, the FEA method can provide estimates of strength that are commonly obtained through mechanical testing, e.g. whole bone stiffness and failure load, as well as other physical properties that are difficult to measure experimentally, e.g. strain distribution and strain energy density distribution. How well the finite element solution approximates the exact biomechanical phenonemon under investigation strongly depends on the quality of the input.

High-resolution digital imaging, including micro-computed tomography (μCT) and high resolution magnetic resonance imaging (HR-MRI), has enabled the creation of finite elements models of trabecular bone that possess an exquisite level of anatomical detail. These "high resolution FEA" or "micro-FEA" models are created by converting each image voxel occupied by bone tissue directly into a cubic finite element *(Hollister SJ et al, 1994; van Rietbergen B et al, 1995)*.

Studies have demonstrated the feasibility and potential utility of combining QCT, HR-pQCT or HR-MRI images with FEA methods to assess the effects of whole bone structure on its mechanical properties *(Pistoia W, 2001 et al; van Rietbergen B et al, 2002; Newitt DC et al, 2002a; Newitt DC et al, 2002b)*.
Improved imaging and computational methods have made subject specific FEA more feasible, and enevitably further technological advances will continue to enhance this capability.
QCT-based FEA allows modeling of the mechanical competence of whole bones under specific loading conditions *(van Rietbergen B, 2001; Crawford RP et al, 2003; Chevalier Y et al, 2007)*. Initial studies have shown that additional information on treatment effects can be obtained *(Keaveny TM et al, 2007)*. It is also possible to derive independent indices of fracture risk *(Bouxsein ML et al, 2006; Melton LJ III et al, 2007)*.
However, prospective studies on risk assessments and clinical guidelines for appropriate use are lacking. Future advances in QCT, specifically for clinical research, will be very substantial.

1.8.3 Radionuclide Scintigraphy in Bone Disease

Radionuclide bone imaging remains the most widely used method for detection of benign and metastatic involvement of the skeleton. Current γ-cameras are able to perform high-resolution imaging in short scan times. More recently, single photon emission CT (SPECT) imaging has become widely available and is becoming routine in nuclear medicine, leading to improved sensitivity and specificity for lesion detection. Currently hybrid SPECT/CT and positron emission tomography (PET)CT scanners are the newest additions to the diagnostic armamentarium.
Methylene diphosphonate (MDP), labeled with ^{99c}Tc, is the most commonly used radiopharmaceutical for bone scintigraphy. The degree of accumulation in bone is dependent of local blood flow but is influenced more strongly by the degree of osteoblastic activity. Pathologic processes that involve bone result in increased bone turnover, with both osteoblast and osteoclast activity being increased.

A detailled description of the use of bone scintigraphy in metabolic bone disease, such as bone metastasis, Paget's disease, hyperparathyreoidism, renal osteodystrophy and osteomalacia, is beyond the scope of this paragraph.
The bone scan has no role in the diagnosis of osteoporosis per se, but is most often used in established osteoporosis to diagnose vertebral or other fractures, e.g. sacral, pelvis, or ribs and has a valuable role in evaluation and management of patients with back pain *(Cook GJR et al, 2002)*. The characteristic appearance of a vertebral fracture is that of an intense, linearly increased tracer uptake at the affected level. Although the bone scan may become positive immediately after a fracture, this is site specific, and in the spine, the bone scan image can take up to 2 weeks to become abnormal *(Spitz J et al, 1993)*. The changes diminish gradually over a period of time, with the scan normalizing between 3 and 18 months after the incident *(Spitz J et al, 1993; Fogelman I, 1980)*.
In a patient with back pain and multiple vertebral fractures on radiographs, but with a normal bone scan, recent fracture can be excluded and other causes of the pain should be considered. Osteoporosis patients with chronic back pain may have unsuspected abnormalities affecting the zygoapophysical (facet) joints *(Cook GJR et al, 2002; Ryan PJ & Ing SW, 1992)*. To indentify abnormalities in these facet joints, SPECT imaging is essential.
Vertebral fracture is defined on the basis of abnormal morphometry *(Cook GJR et al, 2002)*, but morphometric abnormalities are not specific to fracture and may be caused by congenital anomalies. The bone scan may differentiate, whether the morphometric abnormality is related to fracture, provided that it is acquired within several months of the start of symptoms.
Bone scintigraphy has an important role in assessing suspected fractures where radiography is unhelpfull, either because of poor sensitivity (e.g. sacrum, pelvis, rib) of because adequate views are not obtainable because of the patient's discomfort.

1.8.4 Assessment of fracture risk

Viewing the importance, the increasing prevalence and the increasing awareness of osteoporosis, together with the development of treatments of proven efficacy, widespread facilities for the assessment of osteoporosis are required. Measurements of bone mineral density are a central component of any provision, as osteoporosis is defined in terms of BMD and microarchitectural deterioration of bone tissue. There are no satisfactory clinical tools available to assess bone quality independently of BMD. So for practical purposes, the assessment of osteoporosis depends on the measurement of skeletal mass.
The clinical significance of osteoporosis depends on fractures that arise with their attendant morbidity and mortality. Although bone mass is an important component of fracture risk, other abnormalities occur that contribute to fragility. In addition, a variety of non-skeletal factors, such as liability to fall and force of impact, contribute to fracture risk. Moreover, accurate assessment of fracture risk should ideally take into account other readily measured indices of fracture risk, particularly those that add information to that provided by BMD.

1.8.4.1 Assessment of fracture risk by BMD

Many prospective population studies indicate that the risk for fracture increases by a factor 1.5 to 3.0 for each SD decrease in BMD (Marshall D et al, 1996). The ability of BMD to predict fracture is comparable to

the use of blood pressure to predict stroke and significantly better than serum cholesterol to predict myocardial infarction (Kanis JA et al, 2001 (a)).
Accuracy is improved by site specific measurements So for hip fracture, the risk might ideally be measured at the hip. In the immediate postmenopausal population, measurements at any site (hip, spine and wrist) predict any osteoporotic fracture equally well with a gradient of risk of approximately 1.5 per SD decrease in BMD.
The highest gradient of risk is found for hip BMD to predict hip fracture, where the gradient of risk is 2.6. Thus an individual with a T-score of -3 SD at the hip would have a $(2.6)^3$, or greater than 15-fold higher risk than an individual with a T-score of 0 SD. In contrast, the same T-score at the spine would yield a much lower estimate or a 4 fold increase ($(1.6)^3$. This emphizes the importance of accuracy or gradient of risk in the categorization of fracture risk.
Despite the performance characteristics, it should be recognized that, just because BMD is normal, there is no guarantee that a fracture will not occur. A normal BMD implies simply that the risk is lower. Conversely, if BMD is in the osteoporotic range, fractures are more likely, but not invariable. The detection rate for fragility fractures by BMD is low and most of the fractures occur without osteoporosis (Kanis JA et al, 2001a). The low sensitivity is one of the reasons why widespread population-based screening at the time of the menopause, is generally not recommended in women.

1.8.4.2 **Assessment of fracture risk by BMD and age**

The performance of the BMD test can be improved by the concurrent consideration of risk factors that operate independently of BMD. The best example is age. The same BMD T-score at any one site has a different significance at different ages. For any BMD, fracture risk is much higher in the elderly than in the young (Hui SL et al, 1988, Kanis JA et al, 2001b). At the age of 50 year, hip fracture risk was found to be increased by 3.7-fold per SD decrease in femoral neck BMD, whereas at the age of 80 year, the gradient of risk was 2.3 *(Johnell O et al, 2005)*. Thus, the consideration of age and BMD together increased the range of risk that can be identified.

1.8.4.3 Assessment of fracture risk by BMD and other risk factors

A large number of additional risk factors for fracture have been identified. For the purposes of risk assessment, interest lies in those factors that contribute significantly to fracture risk over and above that provided by BMD measurements or age (Kanis JA et al, 2002a). A caveat is that some risk factors are not amenable to particular treatments, so that the relationship between absolute probability of fracture and reversible risk is important. Liability to falls is an appropriate example, where the risk of fracture is high, but treatment with agents affecting bone metabolism may have little effect on risk.
In general, risk factor scores *(Rubin KF et al, 2013)* show relatively poor specificity and sensitivity for prediction of either BMD or fracture risk (Cummings SR et al, 1995; Ribot C et al, 1992; Poor G et al, 1995). Over the past years, a series of meta-analyses has been performed to identify clinical risk factors that could be used in case finding strategies with or without the use of BMD (Kanis JA & Johnell O, 2005).

1.8.4.3.1 Low body mass index (BMI)

A low BMI is a significant risk factor of hip fracture. The fracture risk is nearly 2-fold increased when compared to individuals with a normal BMI (20 to 25 kg/m^2). It is important to note that overweight (BMI 25 to 30 kg/m^2) is not associated with a decrease of risk, i.e. leanness is risk factor rather than obesity being a protective factor. It is also important to note that the value of BMI in predicting fractures is very much diminished when adjusted for BMD.

1.8.4.3.2 History of fragility fracture

Many studies indicate that history of a fragility fracture is an important risk factor for further fracture (Klotzbuecher CM et al, 2000). Fracture risk is approximately doubled in the presence of a prior fracture (Table 2). The increase in risk is even more marked for a vertebral fracture after a previous spine fracture (Kerkeni S et al, 2009). In general, adjustment for BMD would decrease the relative risk by 10-20% **(Table 2)**.

1.8.4.3.3 Familial history of fragility fracture

A family history of fragility fracture in first degree relatives is a significant risk factor, that is largely independent of BMD. Especially, a family history of hip fracture is a stronger risk factor than a family history of other osteoporotic fractures and is independent of BMD.

1.8.4.3.4 Smoking

Active smoking is a risk factor partly dependent of BMD

1.8.4.3.5 Glucocorticoid use

Glucocorticoid intake is an important cause of osteoporosis and fractures (Van Staa TP et al, 2002). The fracture risk conferred by the use of glucocorticoids is, however, not solely dependent on bone loss, and BMD independent risks have been identified.

1.8.4.3.6 Alcohol

The relationship between alcohol intake and fracture risk is dose dependent. Alcohol intake of two units or less daily does not result in increased fracture risk. Intakes of three or more units daily are associated with a dose dependent increase of fracture risk.

1.8.4.3.7 Rheumatoid Arthritis

There are many secondary causes of osteoporosis, e.g. inflammatory bowel disease, endocrine disorders, chronic obstructive pulmonary disease. In most instances, it is uncertain to what extent this is dependent on low BMD or other risk factors such as the use of glucocorticoids. Anyway, rheumatoid arthritis patients show an increased fracture risk independent of BMD and the use of glucocorticoids.

Table 2: Risk ratio (RR) for osteoporotic fractures and 95% CI associated with risk factors adjusted for age, with and without adjustment for BMD (Kanis JA & McCloskey EV, 1996)

	Without BMD		With BMD	
Risk indicator	RR	95% CI	RR	95% CI
Body mass index				
20 vs 25 kg/m^2	1.27	1.16-1.38	1.02	0.92-1.13
30 vs 25 kg/m^2	0.89	0.81-0.98	0.96	0.86-1.08
Prior fracture after 50 yr	1.86	1.72-2.01	1.76	1.60-1.93
Parental history of hip fracture	1.54	1.25-1.88	1.54	1.25-1.88
Current smoking	1.29	1.17-1.43	1.13	1.00-1.25
Every use of systemic corticoids	1.65	1.42-1.90	1.66	1.42-1.92
Alcohol intake > 2 units daily	1.38	1.16-1.65	1.36	1.13-1.63
Rheumatoid arthritis	1.56	1.20-2.02	1.47	1.32-1.92

1.8.4.3.8 Use of FRAX without BMD

A large number of fracture risk assessment tools have been developed. Far from all have been validated in external studies, more of them have absence of methodological and transparent evidence, and few are integrated in national guidelines. Although the literature on this topic is mainly on women, referrals to DXA in men should/could be taken on the same basis.
In a systematic review Rubin et al *(Rubin KG et al, 2013a)* gave an overview of existing valid and reliable risk assessment tools for the prediction of osteoporotic fractures. Only six tools have been tested more than once in a population-based setting with acceptable methodological quality. None of the tools performed consitently better than the others and simple tools (i.e., the Osteoporosis Self-assessment Tool (OST), Osteoporis Risk Assessment Instrument (ORAI), and Garvan Fracture Risk Calculator (Garvan), often did as well as, or better than more complex tools (i.e., Simple Calculated Risk Estimation Score (SCORE), WHO Fracture risk assessment Tool (FRAX®), and Qfracture). No studies determined the effectiveness of tools in selecting patients for therapy and thus improving fracture outcomes. High-quality randomized-design with population-based cohorts with different case mixes is needed.
Generally spoken, a technical investigation should be ordered if the result can influence the treatment. As is the case in the NOGG guideline in UK, DXA referrals are advised in the intermediate absolute risk group, as calculated by FRAX®. The DXA result, integrated in the FRAX® calculator, will then determine if a defined intervention threshold is reached. However, in other countries, e.g. with higher availability of DXA devices, referrals will also depend on the cost and reimbursement conditions of the DXA test, as well as cost and reimbursement conditions of the treatment itself. Also, a specific patient perspective will provide an indication for a DXA investigation, e.g. if patient reassurance is the goal.

1.8.4.4 Biochemical assessment of fracture risk

Biochemical markers of bone turnover (see also paragraph 1.6.2.) are increased after menopause, and in several studies the rate of bone loss varies according to the marker value (Delmas PD et al, 2000a). Prospective studies have shown an association of osteoporotic fracture with indices of bone turnover independent of BMD in both women at the time of menopause and in older women (Johnell O et al, 2002). Data on the fracture prediction by biochemical markers in men are sparce *(Szulc P et al, 2007)*.

1.8.4.5 Fracture probability

The absolute risk of fracture depends on age and life expentancy, as well as on the current relative risk. In general, the remaining lifetime risk of fracture decreases with age, expecially after the age of 70 year, because the risk of death with age outstrips the increasing incidence of fracture with age. Estimates of lifetime-risk are of value in considering the burden of osteoporosis in the community and the effects of interventions. They are less relevant for assessing risk of individuals in whom treatment might be envisaged (Kanis JA et al, 2001b). Therefore, the international osteoporosis foundation (IOF) and the World Health Organisation (WHO) recommend that risk of fracture should be expressed as a shorter-term absolute risk, i.e. probability over a 10-yr interval (Kanis JA et al, 2002b). The 10-year covers the likely duration of treatment. The major advantage of using absolute fracture probability is that it standardizes the output from the multiple techniques and sites used for assessment. The estimated probability will of course depend on the performance characteristics (gradient of risk) provided by any technique at any one site. Moreover, it also permits the presence or absence of risk factors other than BMD to be incorporated in a single metric. This is important because there are many risk factors that give information over and above that provided by BMD and age.

The general relationship between cumulative number of risk factors and probability of fracture is shown in Table 3. For example, a women at the age of 60 has on average a 10-year probability of hip fracture of 2.4%. In the presence of an extra risk factor, e.g. a prior fragility fracture, the risk is increased 2-fold and the probability increases to 4.8%. The integration of risk factors is not new and has been applied succesfully in the management of coronary heart disease (Dyslipidaemia Advisory Group on behalf of the Scientific Committee of the National Heart Foundation of New Zealand, 1996).

Table 3: Relative risk (RR) of fracture in men and women from Sweden according to age and other risk factors compared to the average population (Kanis JA et al, 2001a).

	Age (yr)			
RR	50	60	70	80
Hip Fracture				
RR in Men				
1	0.84	1.26	3.68	9.53
2	1.68	2.50	7.21	17.89
3	2.51	3.73	10.59	25.26
4	3.33	4.94	13.83	31.75
RR in Women				
1	0.57	2.40	7.87	18.00
2	1.14	4.75	15.1	32.00
3	1.71	7.04	21.7	42.9
4	2.27	9.27	27.7	51.6
Hip, clinical spine, humeral or Colles' fracture				
RR in Men				
1	3.3	4.7	7.0	12.6
2	6.5	9.1	13.5	23.1
3	9.6	13.3	19.4	33.9
4	12.6	9.27	24.9	39.3
RR in Women				
1	5.8	9.6	16.1	21.5
2	11.3	18.2	29.4	37.4
3	16.5	26.0	40.0	49.2
4	21.4	33.1	49.5	58.1

Algorithms that integrate the weight of clinical risk factors for fracture risk, with or without information on BMD, have been developped by the WHO Collaborating Center for Metabolic Diseases at Sheffield, UK (Kanis JA, 2008). The risk factors used are given in (Table 4).

Table 4: Clinical risk factors used for the assessment of fracture probability (Kanis JA, 2008).

Age
Gender
Body mass index
Previous fragility fracture, particular hip, wrist, spine including morphometric vertebral frature
Parenteral history of hip fracture
Glucocorticoid treatment (> 5 mg prednisolone daily during 3 months or more)
Current smoking
Alcohol intake > 2 units daily
Rheumatoid arthritis as diagnosed by a physician
Other secondary causes of osteoporosis
Untreated hypogonadism in men and women (e.g. premature menopause, bilateral oophorectomy or orchidectomy, anorexia nervosa, chemotherapie for breast cancer, hypopituitarism)
Inflammatory bowel disease (e.g. Crohn's disease and ulcerative colitis). It should be noted that the risk is in part dependent on the use of glucocorticoids, but an independent risk remains after adjustment of glucocorticoid exposure.
Prolonged immobility (e.g. spinal cord injury, Parkinson' s disease, stroke, muscular dystrophy, ankylosing spondylitis)
Organ transplantation
Type I diabetes
Thyroid disorders (e.g. untreated hyperthyroidism, overtreated hypothyroidism)
Chronic obstructive pulmonary disease

The FRAX tool (www.shef.ac.uk/FRAX) computes the 10-yr probability of hip frature or a major osteoporotic fracture. A major osteoporotic fracture is a clinical spine, hip, forearm or humerus fracture. The probabilities are country specific and based on local epidemiologic data on fracture and mortality. In the absence of local data a surrogate is chosen.
The assessment takes no account of prior treatment nor of dose responses for several risk factors. For example, two prior fractures carry much more risk that a single prior fracture. Dose responses are also evident of glucocortioid use. A prior clinical vertebral fracture carries approximately a 2-fold higher risk than other prior fractures. Because it is not possible to model all such scenarios with the FRAX algorithm, these limitations should temper clinical judgement.
The use of clinical risk factors in conjunction of BMD improves sensitivity of fracture prediction without adversely effecting specificity.

1.8.4.6 The Belgian FRAX® model : its use and proposal for a change in the reimbursement criteria for osteoporosis treatments.

Several guidelines on the management of osteoporosis and fracture risk have been published, including a European guidance (Kanis J et al, 2008b). This guidance highlights the fact that even though a low BMD is strongly associated with fracture risk, different other risk factors are independent contributors to the risk of fracture. Those risk factors, added to the BMD measurement, improve the sensitivity of the indentification of patients at high risk of fracture. In European countries this knowledge is used in a more subjective way by specialists to rationalize approaches to treatment.

The introduction of the FRAX® algorithm has resulted in a more systematic and validaded way to estimate fracture risk. FRAX® models to estimate fracture probability are country-specific. They are calibrated on national data for fracture and death rates. These data have been gathered and validated in the last years in Belgium. The Belgian FRAX® model has been published (Johanson H et al, 2011) and is available on the FRAX-website (www.shef.ac.uk/frax) since 2011.
The number of hits on the Belgian FRAX® website is the highest among other countries (Herlund et al, 2013). The most probable explanation for this observation is that the calculation of the fracture probability based on FRAX is requested for the reimbursement of a DXA examination in Belgium.

However, in Belgium a survey, that has been conducted in general physician's practice, illustrate the still low use of the FRAX® tool (Bruyère O et al, 2013b). The first reason for this is obviously the absence of knowlegde of this tool. The second explanation of the low use is related to the absence of national guidelines on the use of the FRAX® tool and the absence of requirement to calculate FRAX® to have access to drug reimbursement.

Threshold values of absolute 10-year fracture probability for the indication for pharmacological fracture prevention have been proposed in Belgium (Hiligsmann M et al, 2013). It has been demonstrated (Bruyère O et al, 2013a) that the available health care budget could be spent more optimally when drug reimbursement is considered based on age-dependent intervention thresholds proposals derived from the Belgian FRAX® model, compared to the present reimbursement criteria based on BMD and vertebral fracture.

2 Introduction to osteoporosis in men

Studying aging in men is increasingly relevant as longevity in men, as in women, is still increasing. In this context, looking at determinants of bone metabolism in older men is of contributive value, as prospective epidemiological data indicates that senile osteoporosis with fractures in men results in increased suffering and excessive mortality, even more importantly than in women of the same age.
As osteoporosis in men and his related fractures are now been recognized as an important clinical problem, new information is being accumulated on its scope, pathophysiology, evaluation, and treatment. Considering that, studies have provided evidence that treatments which decrease fracture risk in women do the same in men, it is hoped that more men will be evaluated and treated for this often neglected disorder.

Since many years, osteoporosis in men has been the main research topic in the Unit of Osteoporosis and Metabolic Bone Diseases (a collaboration initiative of the departements of Endocrinology and Rheumatology) at the Ghent University Hospital. Next to the extensively reported male aging study in this doctoral thesis, intended to study "hormonal" and "bone" aging in men, several other programs were initiated with a quite extensive clinical reseach team over the years in the Unit. The most important drive to start additional studies, was the insight that osteoporosis in men (in an even more explicite way than in females) is not mainly a sex-hormone deficiency driven disease, as was thought in previous years by Fuller-Albright (1941).

In addition to the changes in sex steroids levels that occur with aging in both men and women, sex steroid-independent factors also occur, including reductions in the production of growth factors important for osteoblast differentiation and function. With aging, both the amplitude as the frequency of growth hormone pulsatile secretion is diminished (Ho KY et al, 1987), leading to a decreased hepatic production of both IFG-1 and IGF-2, an effect that may contribute to decreased bone formation with aging (Boonen S et al, 1999).
Additionally, aging is associated with increased levels of the IGF inhibitory protein, IGFBP-2, which also correlates inversely with bone mass in elderly subjects (Amin S et al, 2004).
Finally, it is likely that there are intrinsic changes in osteoblast and perhaps osteoclast lineage cells as a consequence of the aging process (Moerman EJ et al, 2004).
In older people calcium intake and absorption are often deficient. In combination with the frequent vitamin D deficiency, the negative calcium balance can induce a secundary hyperparathyroidism. This leads to increased bone turnover and bone loss by the increased activation frequency of remodeling cycles with a negative bone balance.

A progressive decline in physiologic reserves inevitably occurs with aging. The resulting frailty is referred to as the frailty syndrome and is widely recognized in geriatric medical practice (Gielen E et al, 2012). Frailty results from reaching a threshold of decline across multiple organ systems. By consequence, frail elderly experience an excess vulnerability to stressors and are at high risk for functional deficits and comorbid conditions, which can lead to institutionalization, hospitalization and death (Walston J et al, 2006). Although frailty affects both musculoskeletal and non-musculoskeletal systems, sarcopenia, which is defined as age-related loss of muscle mass, force and power (Baumgartner RN et al, 1998), constitutes one of the main determinants of fracture risk in older age and one of the main components of the clinical frailty syndrome. As a result, operational definitions of frailty and therapeutic strategies in older patients tend to focus on the consequences of sarcopenia. These are disability (Janssen I et al, 2006), loss of mobility (Visser M et al, 2005), impaired balance (Szulc P et al, 2005), increased risk of falls (Landi F et al, 2012a), fractures (Lang TF et al, 2010) and death (Landi F et al, 2012b).

As said previously, the main forces on the bone originate from muscle activity (due the system of leverarms) and not from gravity. Referring to the principle of the bone mechanostat, sarcopenia is inevitably followed by bone loss. The forces applied to the bone in sarcopenic subjects decrease, and the bone mass and strenght will adapt downwards following the progression of the sarcopenia to reach a new steady state within the set-points of the strain levels. Bone loss in older people is, in this respect, not only or directly due to the changes in hormonal levels, poor nutrition, lack of vitamin D and other contributing factors, but is likely due for an important part to mechanical factors resulting from changes in muscle

function. However, the latter changes might in turn be influenced by factors such as hormonal changes, protein intake or vitamin D deficiency. As sarcopenia (and frailty as a whole) is also a risk factor for falls, besides inducing bone loss, it is an important factor contributing to the exponential increase of the fracture incidence with aging.

In addition to the general introduction, it was thought to be useful, in respect to the specific questions addressed is this thesis, to provide a general overview on male osteoporosis. This is done extensively in the included co-authored review "Osteoporosis in Men".

Chapter 1

1. Osteoporosis in men

Jean-Marc Kaufman, Stefan Goemaere

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Chapter 2 Aims and outline of the thesis

1 General aims of the thesis

Previous work of Prof. Dr. Em A. Vermeulen demonstrated first in 1968, that serum testosterone could be measured in serum *(Vermeulen A & Verdonck L, 1968)* and that the levels were declining with age in men *(Vermeulen A et al, 1972)*. Since then, aging in male has been a favorite research interest of the department of Endocrinology at the Ghent University Hospital. Consequently, the hypothesis that this decline was responsible for some clinical aspects of aging in men was generated and this formed the basis for a number of pilot studies.
Although the main focus of the Merelbeke Study was on bone metabolism, a number of extraskeletal aspects of health were considered. We mention analyses on body composition, hormonal aspects, testicular function, genetic determinants, quality of life, aging male symptoms, depression, muscle force, physical performance, mortality and vitamin D. These data were and are still subjects of other doctoral programs: *Crabbe P et al, 2006; De Buyser S et al, 2013; Lapauw BM et al, 2007, 2008; Mahmoud AM et al, 2000, 2003; Taes YE et al, 2006; T'Sjoen G et al, 2004, 2005; Van Pottelbergh I et al, 2001a, 2001b, 2002; Vanbillemont G et al, 2009; Vermeulen A et al, 1999a, 1999b, 2002*.

In contrast to many of these general health parameters, aspects of bone metabolism can be measured more precisely and accurately. Therefore, in the Merelbeke Study, bone has been chosen as a target organ to investigate the effects or related changes in sex steroid levels including possible threshold levels of serum sex steroids below which bone was adversely affected.

The subject of this thesis is to explore the value of different assessment techniques of bone metabolism and bone mineral status in older males. The main focus is on their relationship with sex steroids. The global purpose of the cross-sectional and longitudinal evaluation is to provide arguments for or against the existence of an aging related male hormonal deficiency state affecting the skeleton.

2 Outline of the thesis

The results of the performed studies are presented in Chapter 3, 4 and 5
Chapter 3.1 and 3.2 describes the DXA methods used.
Chapter 4 concerns the cross-sectional analyses of the Merelbeke Study
Chapter 5.1, 5.2 and 5.3 pertain to findings in the longitudinal evaluations in the Merelbeke Study

2.1 Assessment of osteoporosis in men

One of the main problems with DXA values obtained with different devices is that these cannot directly be compared. The absolute values (g/cm^2) obtained on equipment from different manufacturers i.e. Hologic, Lunar and Norland are different, because of differences in data acquisition techniques, calibration and bone-edge detection algorithms. From these absolute values, however, T-scores will be calculated using different manufacturer-derived databases.
To avoid these inconsistencies and to provide a uniform basis for patient assessment in Belgium, the Belgian national osteoporosis society (Belgian Bone Club (BBC)) implemented a uniform expression of

BMD in Belgian postmenopausal females. The current study aimed at pursuing a similar approach in men, to establish uniform thresholds for the diagnosis of male osteoporosis.
Detailed report of the study is given in the section Results – chapter 3.1.

Senile osteoporosis in men is now recognized as a significant public health problem. As in women, bone mass assessments in men can be of interest for health care. In women, bone mass assessment (BMD) by dual-energy X-ray absorptiometry (DXA) at the spine and at the proximal femur is a well-established standard to diagnose postmenopausal osteoporosis and to guide its prevention and treatment.

Compared to women, in men only few studies focused on the relationship between bone mass density (BMD) and fracture risk. The BMD criteria that should be used to identify osteoporosis in men are still matter of debate.
Peripheral DXA has been proposed as a surrogate for measurement of the central skeletal sites, the spine and the hip. In addition quantitative ultrasound (QUS) techniques for the assessment of bone properties offer an attractive possible alternative to the central DXA assessments. QUS techniques are radiation free, relatively cheap and the equipment is easily transportable. However, there is only limited experience with the use of peripheral DXA and QUS techniques in older men.
The aim of the present cross-sectional study was to compare the performance of bone assessments by DXA and QUS at different peripheral sites of the skeleton.
Detailed report of this study is given in the section Results – chapter 3.2.

2.2 Cross-sectional relationship between bone density, bone turnover indices and sex steroids in older men

The relationship between bone mineral density (BMD) and fracture risk in older men is remarkably similar to that in postmenopausal women.
However, little is known about the mechanisms that underlie senile bone loss in men.
Aging in healthy men is accompanied by a progressive, albeit variable, decline in the levels of both serum testosterone and bioavailable estradiol. It is not known whether relative sex steroid deficiency described in older men affects bone mineral mass or density. The pathophysiological mechanisms that underlie the cross-sectional and eventual longitudinal relationships between sex steroid status and BMD in older men remained to be established **(Figure 31)**.

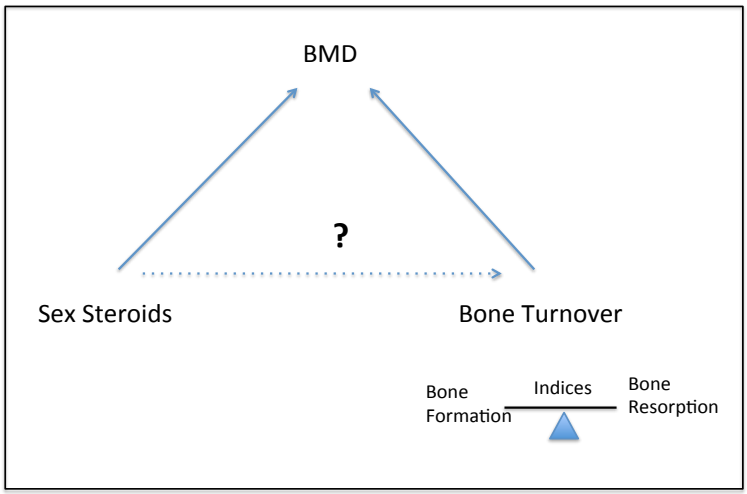

Figure 31: Estimated causality and direction of the reported association between serum sex steroid levels, bone mineral density (BMD) and bone turnover indices of bone formation and bone resorption.

The purpose of the cross-sectional study was to determine whether lower BMD was associated with higher values for biochemical indices of bone turnover in a well-characterized group of community-dwelling, ambulatory, older men, and also to investigate the role of sex steroid status in the determination of bone turnover in this study population **(Figure 30)**.
Detailed report and conclusions of this study is given in the section Results – chapter 4

2.3 Longitudinal assessments of bone density and bone geometry in relation to bone turnover indices, sex steroids and genetic markers in older men.

Aging in men is accompanied by a progressive inter-individually variable decline in serum levels of bioactive sex steroids and this has been implicated in their clinical phenotype of bone loss.
An additional investigation consisted of a longitudinal study where BMD as well as sex steroid status in the same group of community-dwelling, ambulatory, older men, was explored at yearly intervals during a 4-year period.
In addition, attention was paid to the possible modulation of estrogen effects by a tetranucleotide (TTTA)n –repeat polymorphism of the CYP19 gene. The gene CYP19, located on chomosome 15q21.1, encodes the aromatase enzyme.
The conclusions of this study are reported in the section Results – chapter 5.1

In the above mentioned studies the associations of serum sex steroid levels with prevalent areal BMD and bone loss in men as assessed by (DXA) in cross-sectional and longitudinal studies in older men.
However, BMD in itself is not a direct measure of bone strength and the relation between serum sex steroids and fracture risk appears to persist after adjustment for BMD in older men.
So, additional explanations need to be explored for this relationship.
Assessing other variables, BMD-independent skeletal features of bone quality, e.g. skeletal geometry, may have a role and may capture part of the variation in fracture risk.
Therefore, we investigated in a population of older men the associations of sex steroids with bone geometrical parameters of the femoral neck as assessed by the Hip Structural Analysis methodology using DXA, in both a cross-sectional and longitudinal setting.
The conclusions of this study are reported in the section Results – chapter 5.2

Telomeres are regions of repetitive nucleotide sequences at each end of a chromatid, which protect the end of a chromosome from deterioration or fusion with neighbouring chromosomes. With every round of cell division distal telomere sequences are lost thereby ultimately limiting the potential of cell replication. Telomere ends thus keep track of the replicative history of somatic cells and register biological aging - not a priori similar to chronological aging - at the tissue level, and telomere end length thus can play a predictive role in clinical age-associated phenotypes and disorders.
Interestingly, estrogens are suggested to influence telomere length dynamics by the activation of telomerase, the enzyme that counteracts loss of telomeric sequences during DNA replication.
Therefore the aim of a study in the healthy older men of the Merelbeke Study population is to assess the association of mean peripheral blood leucocyte telomere length with sex steroid status, BMD status and prospectively assessed BMD changes in this population.
We reported the conclusions of this study in the section Results – chapter 5.3

3 The study population in the thesis

3.1 The healthy young adult male reference population

Standardizaton of absolute values between different manufacturers of bone densitometry is essential for the uniformisation of the diagnostic categorization along the availability of Hologic, Lunar or Norland devices.
A population of 229 young healthy men was recruited in 8 Belgian university and peripheral DXA centers representing an adequate sample for the Belgian population.
Normality data were produced for Hologic and Lunar devices. As in Belgium only a few Norland DXA devices were available an inadequate number of patients was expected and consequently these DXA centers were not invited for the study.

3.2 The Merelbeke Study population

In order to set up a powered prospective study to find evidence for the existence of clinical consequence of hormonal changes in aging male, a pilot study in the municipality of Merelbeke was performed to assess the expected range and changes of bone mineral density (BMD) and sex steroid levels in older men aged 70 to 80 years. It was calculated that for estimated BMD changes of 1% per year, a study of 300 older males over a time period of 4 years was needed to demonstrate relationships between bone loss with the decay of serum levels of sex steroids.

To recruit the cross-sectional and longitudinal follow-up study population the older ambulatory men were invited to participate in the by a written invitation letter. In 1996 the study subjects were recruited from the population register of the semirural community of Merelbeke with 22,000 inhabitants, near the Ghent University Hospital (Belgium). Age between 70 and 85 years was the only initial selection criterion. From the 748 inhabitants in the age group, 352 (47.1%) signed an informed consent for participation in longitudinal observational study for 4 years, as approved by the ethics committee of the Ghent University Hospital. Sixty-nine subjects (19.6%) were excluded from the cross-sectional baseline analyses due to disease and/or treatment with potentially substantial impact on bone mineral metabolism or hormonal parameters **(Figure 32)**.

All study subjects who started the study (n=352) were invited for yearly evaluation in a temporarily installed examination room located centrally in the village. These visits were done from 1996 to 2000. For the longitudinal analyses all available data were used after applying identical exclusion criteria as for the baseline assessment. For longitudinal assessment of BMD changes and aromatase polymorphism analysis, data on 214 subjects were available. For longitudinal evaluation of femoral neck geometry parameter changes from baseline to the 4th year, data of 147 subjects could be used. Telomere restriction fragment length was assessed on a subsample of 110 subjects at baseline. From those 84 were available for longitudinal BMD data analysis. The difference in the subject number for longitudinal analyses is due to the availability of complete datasets of all variable. Specific exclusion criteria or reasons for lack of follow-up can be found in the specific sections in the manuscripts.

After the year 2000 the follow-up of these aging subjects until present was limited to yearly postal questionnaires and telephone contacts if necessary. The items followed were general health aspects, falls, fractures and death. All data derived after 2000 are subject of ongoing doctoral work *(De Buyser S, 2013)*.

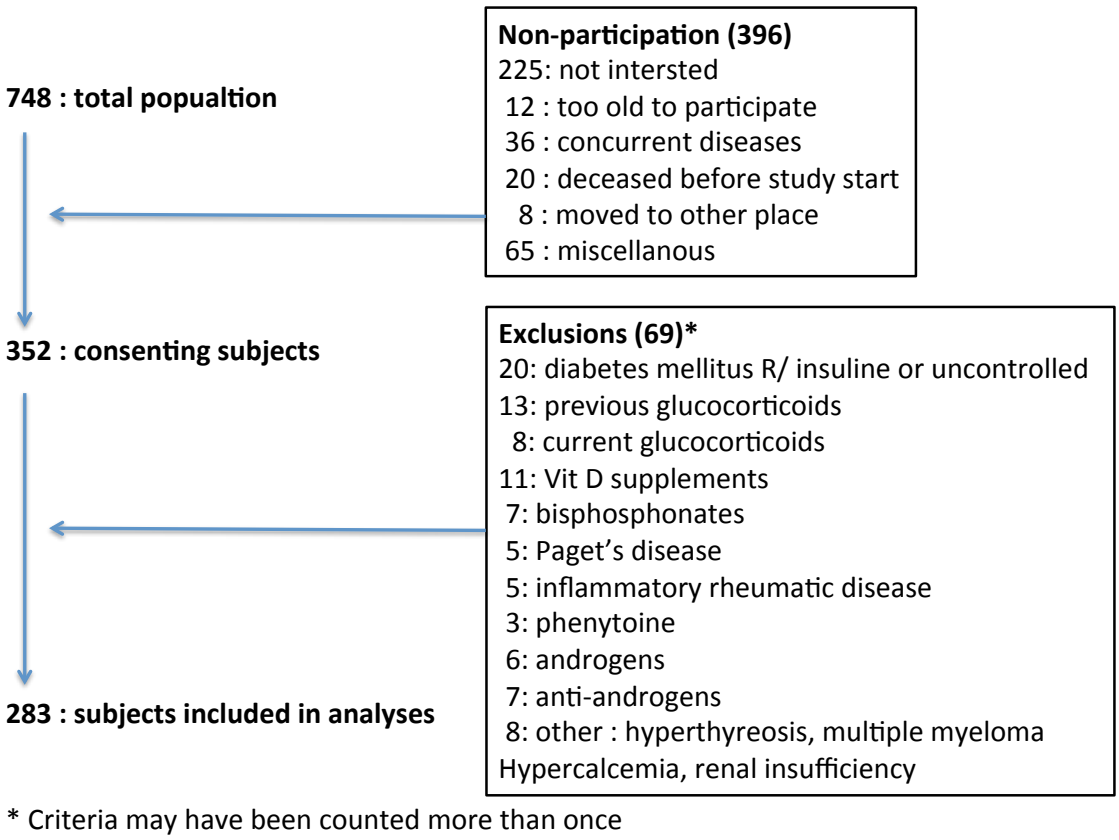

Figure 32: Representation of the Merelbeke study population of older men aged 70 to 85 years of age.

3.3 The reference populations for the Merelbeke Study

As a reference for the "older men group", two groups of younger and healthy male reference subjects were recruited. A considerable number of sons (n=66) of the older men in the Merelbeke Study consented to participate in a similar program as their fathers. This population has been referred to as the middle-age reference (median age 43 years). A second reference population (n=70) of young adult male volunteers (median age 27 years) was recruited from the hospital staff and students.

Chapter 3 to 5

Results

Chapter 3

1 Dual energy X-ray absorptiometry-based assessment of male patients using standardized bone density values and a national reference database.

Stefan Goemaere, Dirk Vanderschueren, Jean-Marc Kaufman, Jean-Yves Reginster, Yves Boutsen, Stefaan Poriau, Joris Callens, Frank Raeman, Geneviève Depresseux, Herman Borghs, Jean-Pierre Devogelaer, Steven Boonen.

On behalf of the Belgian Bone Club (BBC)
and
the Network on Male Osteoporosis in Europe (NEMO).

J Clin Densitom 2007; 10: 25-33.

Chapter 3

2 Ability of peripheral assessments to predict areal bone mineral density at the hip in community-dwelling elderly men.

Stefan Goemaere, Hans Zmierczak, Inge Van Pottelbergh, Jean-Marc Kaufman

J Clin Densitom 2002; 5: 219-228.

Chapter 4

1 Inverse association between bone turnover rate and bone mineral density in community-dwelling men over 70 years of age : no major role of sex steroid status.

Stefan Goemaere, Inge Van Pottelbergh, Hans Zmierczak, Kaatje Toye, Marjan Daems, Rein Demuynck, Hilde Myny, Dirk De Bacquer, Jean-Marc Kaufman.

Bone 2001; 29: 286-291.

Chapter 5

1 Bioavailable estradiol and an aromatase gene polymorphism are determinants of bone mineral density changes in men over 70 years of age.

Inge Van Pottelbergh, Stefan Goemaere, Jean-Marc Kaufman.
(IVP & SG equally contributed to the manuscript)

J Clin Endocrinol Metab 2003; 88: 3075-3081

Chapter 5

2 Estradiol levels modulate longitudinal changes in femoral neck geometry favouring bone strength in community-dwelling men over 70 years of age.

Stefan Goemaere, Hans Zmierczak, Dirk De Bacquer, Bruno Lapauw, Youri Taes, Kaatje Toye, Tom Beck, Jean-Marc Kaufman.

Osteoporosis Int 2014; submitted.

Estradiol levels modulate longitudinal changes in femoral neck geometry favouring bone strength in community-dwelling men over 70 years of age.

STEFAN GOEMAERE[1], HANS ZMIERCZAK[1], DIRK DE BACQUER[2], BRUNO LAPAUW[1,3], YOURI TAES[3], KAATJE TOYE[3], THOMAS J BECK[4], JEAN MARC KAUFMAN[1,3]

1 *Unit for Osteoporosis and Metabolic Bone Diseases*
Departments of Endocrinology and Rheumatology
Ghent University Hospital (9K12 IE)
De Pintelaan 185, Gent, Belgium

2 *Department of Public Health*
Ghent University (Blok A)
De Pintelaan 185, Gent, Belgium

3 *Department of Endocrinology*
Ghent University Hospital (9K12 IE)
De Pintelaan 185, Gent, Belgium

4 *Beck Radiological Innovations, Inc.,*
1450 South Rolling Road
Halethorpe, MD 21227, USA

Conflicts of interest:

Stefan Goemaere, Hans Zmierczak, Dirk De Bacquer, Bruno Lapauw, Youri Taes, Kaatje Toye, and Jean Marc Kaufman declare that they have no conflict of interest.
Thomas J Beck is CEO of Beck Radiological Innovations, a company developing products in radiation protection and x-ray imaging

Corresponding address:

Dr. Goemaere Stefan
Unit for Osteoporosis and Metabolic Bone Diseases
Departments of Endocrinology and Rheumatology, Ghent University Hospital
De Pintelaan 185 (9K12 IE)
9000 Ghent
Belgium
T: +32 9 3324150
F: +32 9 3323817
E: stefan.goemaere@ugent.be

Keywords:
Male osteoporosis, bone mineral density, bone geometry, estradiol, testosterone, longitudinal study.

Abstract :

Introduction :
Hip Structural Analysis (HSA) using dual X-ray absorptiometry (DXA) data provides data on areal bone mineral density (aBMD) and bone geometry. Both are predictors of fractures in both women and men. The present study describes the effects of serum estradiol levels on HSA and aBMD in elderly men.

Methods:
To assess determinants of bone loss, community-dwelling elderly men aged 70 to 85 years were recruited at a population level for a longitudinal follow-up study of 4 years . At annual visits health questionnaires, fasting blood sampling and DXA at the proximal femur were performed. Men with secondary causes for bone changes were excluded from the analyses.

Results:
In this prospective cohort study of elderly men E_2 is associated, not only with aBMD ($r=0.21;p<0.001$), but also with cross-sectional area ($r=0.18;p<0.01$), section modulus ($r=0.13;p<0.05$), endocortical diameter ($p=-0.13;p<0.05$), average cortical thickness ($r=0.21;p<0.001$) and buckling ratio ($p=-0.20;p<0.001$) at the narrow femoral neck. In the cross-sectional baseline analyses, higher serum E_2 levels were correlated with more favourable femoral neck bone strength surrogates at this skeletal site. Prospectively in multivariate analysis, higher serum E_2 levels were significantly associated with a more

favourable evolution of aBMD, average cortical thickness, section modulus and buckling ratio. Standardized regression coefficients were respectively : 0.241, 0.349, 0.243 and -0.242)
Conclusion:
The present study provides evidence that higher E_2 levels in elderly men are associated not only with a better preservation of aBMD but also with a more favourable evolution of HSA derived bone morphometric characteristics, which in turn might contribute to preservation of bone strength independently of aBMD

Mini-abstract:
Higher serum E_2 levels in elderly men are associated with a better preservation of areal BMD, but also with more favourable evolution of HSA derived bone geometry at the femoral neck, which might contribute to preservation of bone strength, independently of areal BMD.

Introduction :
Sex steroid deficiencies have been reported to contribute to fragility fracture risk in older men (1). A majority of studies in men reports a stronger association of increased fracture risk with lower serum estradiol (E_2) than with lower testosterone (T) levels (2-5), although there have been discordant findings (6,7). These data regarding fracture risk are in agreement with the observations of associations of lower serum E_2 levels with lower areal bone mineral density (aBMD) and faster bone loss in men as assessed by dual-energy X-ray absorptiometry (DXA) in cross-sectional (8-11) and longitudinal studies (12-15).
Because BMD itself is not a direct measure of bone strength and the relation between E_2 and fracture risk appears to persist after adjustment for aBMD in elderly men (5) and postmenopausal women (16), additional explanations need to be explored. In this respect, co-morbidities simultaneously affecting E_2 levels and fracture risk, or E_2 non-skeletal tissue effects on neuromuscular function and/or fall risk have been proposed.
Measurements of bone geometry and volumetric BMD in cortical and trabecular bone could contribute to explain the effects of sex steroids on fracture risk not captured by conventional aBMD by DXA (17-20). We previously reported that serum E_2 is positively associated with prevalent aBMD and with a lesser decline of aBMD (14) in a study cohort of generally healthy men of 70 to 85 years of age.
In this cohort, we now present cross-sectional and longitudinal associations between sex steroid levels and aBMD and geometrical parameters of the narrow femoral neck, as assessed by the Hip Structural Analysis (HSA) methodology using DXA (21,22). These DXA parameters have been found to be predictors of fractures in women and men in large prospective cohort studies (20, 23-26). They are strongly genetically determined (27), sex-, age- and body composition-dependent (28,29), sensitive to pharmaceutical interventions for osteoporosis (30-32) and postmenopausal estrogen substitution (33). More recently, dimensional differences assessed by HSA have been proposed to contribute to racial and ethnic variability of fracture rates (34,35).

Materials and methods

Subjects

All non-institutionalised elderly men, aged 70 to 85 year (n=748), from a semi-rural community with 22.000 inhabitants (Merelbeke) near the Ghent University Hospital, were invited to participate in a prospective observational study on the relationship between their sex steroid status and longitudinal bone changes. Informed consent was obtained in 352 subjects; representing a participation rate of 47.1%. Subjects were invited for annual visits during up to 4 years. Self-administrated questionnaires, including medical history and current medical conditions, lifestyle factors (smoking, alcohol, physical activity) and calcium intake, surgical procedures and medication intake, were completed and cross-checked by the study physician. Participants at baseline and follow-up were excluded for analysis in the presence of diseases and/or treatments with potential substantial impact on bone metabolism or hormonal status as described earlier (16). Briefly summarized, exclusion criteria were: current or previous long lasting (> 3 months) systemic glucocorticoid treatment; vitamin D supplementation; insulin treated diabetes; treatment with phenytoin, androgens or anti-androgens; orchiectomy; use of bisphosphonates; Paget 's disease; uncontrolled hyperthyroidism; adrenal insufficiency; multiple myeloma; inflammatory rheumatic diseases; diseases potentially leading to intestinal malabsorption, calcemia > 2.65 mmol/L; fasting glycemia > 8.33 mmol/L or serum creatinine > 177 μmol/L.

Bone mineral density and geometry by Hip Structure Analysis

Dual energy X-ray absorptiometry (DXA) of the proximal femur was performed at baseline and the 4 consecutive annual visits on a Hologic QDR 1000+ scanner, using pencil beam technology (Bedford, MA, USA). The Hip Structural Analysis (HSA) program has been described previously (21,23). Archived scans were analysed at the John Hopkins University. Cross-calibration was conducted using a dedicated phantom provided by TJB. In brief, the program algorithm determines BMD and geometry within narrow regions corresponding to thin cross-sectional slabs of bone viewed on edge. For the present analysis only data of the narrow femoral neck was used. The femoral neck region across its narrowest point is narrower and located more proximally than the standard Hologic neck region, however aBMD trends are comparable (29). Profiles of bone mass were derived from one bone margin to the other and then averaged along the 5-mm length of the region. Variation coefficients (%CVs) of the derived parameters were assessed by duplicate measurement of baseline scans with repositioning.

The following parameters were obtained: areal bone mineral density measured in the standard manner (aBMD; gr/cm^2, %CV: 2.95) and estimates of bone geometry. The width (W) or periosteal diameter (PD) was computed as the blur-corrected distance between the profile margins (W in cm; %CV: 1.91). The bone cross-sectional area, an index of resistance to axial loading, was computed as the profile integral divided by the effective density of bone mineral in fully mineralized bone tissue, being 1.053 g/cm^3 (CSA in cm^2, %CV: 3.26). The cross-sectional moment of inertia was derived from the integral of mass times the square of the distance from the centre of mass, divided by 1.05 (CSMI in cm^4; %CV: 5.00). The section modulus was computed as the ratio of CSMI to the distance from the centre of mass to the nearest outer margin and is a measure of maximal bending strength (SM in cm^3, %CV: 5.09). The estimated endocortical diameter is calculated from W and CSA (ED in cm, %CV: 2.62). The estimated average cortical thickness was derived using simple models of cross-sections as hollow annuli (avCT in cm, %CV: 3.10). For the narrow neck region model it was assumed that a fixed 60% of the neck mass was in the cortex, with the space within filled with the mass remainder as trabecular bone. The buckling ratio is an index of cortical instability under compression and is defined as the ratio of the distance from the centre of mass to nearest outer cortical margin to the mean cortical thickness (BR, dimensionless; %CV: 4.90).

Blood sampling

Venous blood sampling was performed between 8:00 and 10:00 am after overnight fasting. At baseline and at yearly intervals a limited biochemical screening panel was carried out to check for exclusion criteria. Serum samples were stored at -80° C until analysis. Serum sex hormone levels were determined by commercially available immuno-assays, as previously described (18); testosterone (T) (Medgenic, Biosource, Fleurus, Belgium); estradiol (E_2) (DiaSorin s.r.l., Saluggia, Italy, according to an adapted protocol with doubling the amount of serum) and sex hormone binding globulin (SHBG) (Orion Diagnostica, Espoo, Finland). The detection limit for the T and E_2 assay was 10 ng/dl and 0.3 ng/dl, respectively. The inter-assay %CVs were 5.96% and 4.46%, respectively. Bioavailable serum testosterone (BioT) and estradiol (BioE_2) were calculated from total hormone levels, SHBG and albumin

concentrations using a validated equation derived from the mass action law (36,37). Commercial radioimmunoassays were used to determine 25-hydroxyvitamin D (25OHD) (Incstar Corporation, Stillwater, Minnesota, USA) and parathyroid hormone (PTH) (Nichols Institute Diagnostics San Juan Capistrano, CA, USA), insulin growth factor 1 (IGF-1) (IRMA diagnostics after extraction) and IGF-Binding protein 3 (IGF-BP3) (IRMA diagnostics, Webster, TX, USA).
The panel of biochemical indices of bone turnover consisted of serum osteocalcin (OC: N-MID™ Osteocalcin; Osteometer Bio Tech A/S, Herlev, Denmark), serum bone specific alkaline phosphatase (BsAP: Tandem®-R Ostase; Hybritech INC, San Diego, USA), serum C-terminal telopeptide of type I collagen (sCTX: Elecsys® β-CrossLaps/serum, Roche Diagnostics GmbH, Mannheim, Germany). All intra- and inter-assay %CVs were below 10 and 15% respectively. All assays were run using the same lot of reagents/assay kits. Data were reviewed for outliers. No values were excluded from the analysis.

Anthropometry and physical performance tests
Standing height without shoes was assessed with a measuring ruler, weight in light clothes was measured on a digital balance. *Grip strength at the dominant hand was measured using an adjustable hand-held standard grip device (Smedley type hand dynamometer, Baseline®; Smith&Nephew Rolyan Inc, Germantown, USA). The maximum value (kg) of two consecutive measurements was used for analysis. CV calculated from the duplicate measurements was 9%. Timed up and go test (TUGT) was performed as described (38).*

Statistical methods
Spearman correlations were used to evaluate the association between the HSA parameters, age, weight, height, BMI, and sex steroid levels, biochemical markers of bone turnover and functional tests. Multiple linear regression analysis was performed to study the associations in a multivariate framework. The longitudinal changes in the bone parameters were expressed as %changes between the baseline and the final visit after 4 years. The level indicating statistical significance was set at $\alpha < 0.05$. All statistical analyses were performed using SAS software.

Results

Description of the baseline population and follow-up cohort :
After exclusions, 269 men were retained for cross-sectional analyses at baseline. The longitudinal follow-up cohort consists of all eligible subjects with a 4th year time endpoint visit (n=147). Reason for drop-out during the 4 year follow-up time was death (n=62), withdrawal of consent (n=13), poor general health condition (n=18); institutionalisation and transport problems (n=15); initiation of systemic glucocorticoid (n=6) or anti-androgen treatment (n=2); development of uncontrolled diabetes (n=2; fasting glycemia > 8.33 mmol/L), decreased kidney function (n=2; serum creatinine of > 177 µmol/L) or suppressed TSH (n=2, TSH < 0.05 mU/L). Table 1 summarizes the characteristics of the population for cross-sectional analyses and the baseline data for the follow-up subgroup. This table illustrates the similarity of the baseline characteristics concerning age, weight, length, smoking status, functional tests, serum sex steroids levels and bone metabolism parameters. The baseline cross-sectional and the longitudinal HSA parameters at the narrow neck of the proximal femur are shown in table 2.

Univariate associations of femoral neck morphometric characteristics
Age was negatively associated with aBMD, CSA, SM, avCT (r: ranging from -0.16 to -0.20; $p< 0.01$) and positively with BR (r: 0.16; $p<0.05$). No significant correlations were found between age and periosteal or endocortical diameter.
Weight was strongly positively correlated with aBMD, CSA, SM and avCT (r: ranging from 0.34 to 0.47; $p< 0.001$) and weakly associated with ED (r = 0.18; $p<0.01$). The relationships between the HSA parameters with height were similarly correlated as for weight, however generally at lower correlation values. BR was negatively correlated to weight (r: -0.18; p<001) and was not related to height. Longitudinal HSA data (%change from baseline) were not related to age, weight or height. No associations were found for the smoking status, calcium intake and lifetime activity, neither in the cross-sectional nor the longitudinal assessment.
In contrast to the absent or only weak baseline associations of T with HSA parameters adjusted for age, weight and height, E_2 levels showed stronger correlations. These were positive for BMD, CSA, SM and avCT and negative for ED and BR (Table 3). Significant relationships between SHBG levels and geometrical bone parameters were observed in the inverse direction of T and E_2. Consequently, the baseline relationships were stronger when considering the bioavailable fractions of the sex-steroids, particularly for bioT. Neither baseline T, bioT, nor SHBG were significantly associated with any of the %changes of the HSA parameters over the 4 years of follow up (Table 3). In contrast, baseline E_2 was significantly correlated with the %change of BMD,

CSA, SM, avCT and with BR. However, for $BioE_2$ levels only a weak correlation with %change in SM was observed.

Cross-sectional values (Figure 1) of and longitudinal changes (Figure 2) in HSA parameters were explored by quartiles (Q) of E_2. In the cross-sectional baseline analysis E_2 levels above the median were related to mechanically more favourable HSA values, except for ED which did not show a significant correlation (Figure 1). In the longitudinal study over 4 years Q4 is associated with an increase from baseline of the SM (+3%) in contrast to decreases or no significant changes (-0.5 to -1.2%) in the 3 lower quartile groups. The %changes in aBMD, avCT and BR showed significant trends towards better maintenance of mechanical competence in those participants with the highest baseline E_2 quartiles. Although not statistically significant, the same trends were observed for CSA and ED (Figure 2).

Baseline SHBG, IGF-1 and IGF-BP3 levels were not related to baseline hip morphometric parameters, nor to longitudinal changes of them. Age, weight and height adjusted 25OH vitamin D levels were weakly positively correlated with baseline aBMD ($r=0.13$; $p<0.05$) and avCT ($r=0.13$; $p<0.05$) and inversely related to baseline BR ($r=-0.14$; $p<0.05$). No relationships of 25 OH vitamin D were found with longitudinal changes in HSA parameters. PTH was related neither with baseline nor with longitudinal femoral neck HSA assessments.

Bone turnover (BT), as assessed by bone specific alkaline phosphatase (BsAP), osteocalcin (OC) or serum CTx (sCTX), was significantly negatively associated with baseline aBMD (r: -0.12 to -0.24), CSA (r= -0.14 to -0.21) and avCT (r: -0.12 to 0.23). ED and BR correlated positively with baseline BT, except for BsAP. The p-values ranged from <0.05 to <0.001 by Spearman correlation adjusted for age, weight and height. No relationships of BT with the longitudinal assessments were observed.

Time Up and Go Test (TUGT) as assessment of physical performance (higher TUGT indicates lower performance), was negatively related to age, weight and height adjusted aBMD ($r= -0.16$; $p<0.01$), CSA ($r= -0.15$; $p<0.05$), SM ($r= -0.15$; $p<0.05$), avCT ($r= -0.16$; $p<0.01$) and positively with BR ($r=0.16$; $p<0.01$). When relating baseline TUGT with the longitudinal assessments of HSA parameters, significant findings were limited to %change in SM ($r= 0.18$; $p<0.05$). No relationships in cross-sectional or longitudinal analyses were found with baseline smoking status (never/former/current), calcium intake or with grip strength.

<u>Multivariate analyses of femoral neck morphometric characteristics</u>

A multivariate model was constructed including age, weight, height, T, E2, SHBG, sCTX and TUGT. In the cross-sectional analysis (Table 4a) weight was the most important determinant for the baseline morphometric HSA variables, except for ED. For ED height was the most dominant contributor. Weaker independent contributions were provided by TUGT and E_2 levels for aBMD, CSA, avCT and BR. Better physical fitness and higher E_2 levels were associated with more favourable narrow femoral neck bone strength surrogates. No interaction between E_2 and TUGT could be identified (data not shown). Increased bone turnover as assessed by sCTX was significantly contributing to increased ED and BR values (Table 4a).

For the longitudinal changes in HSA variables (Table 4b) baseline E_2 was maintained as the only significant determinant. Higher E_2 levels were associated with a smaller %decrease in aBMD, SM, avCT and with a smaller %increase in BR. The only exception to this general finding was the negative correlation of T with the %change of SM

In this model, SHBG levels were not related to the HSA outcome parameters; neither in cross-sectional-, nor in longitudinal assessment.

Discussion:

The present study illustrates that E_2 is associated not only with aBMD, but also with morphological variables at the narrow femoral neck as assessed by HSA in a population of community-dwelling elderly men aged between 70 and 85 years at baseline. Both in the cross-sectional baseline analysis as well as in the longitudinal assessment, the data show a favourable effect of E_2 on the bone strength surrogates at the femoral neck. Although there is only a limited decrease of serum estrogens with aging in men (39), we found that higher serum E_2 levels, within the observed physiological variation in elderly men, were prospectively associated with a significantly more favourable evolution of aBMD, avCT, SM and BR. This extends earlier findings of positive effects of higher serum E_2 on maintenance of aBMD in elderly men (12-15) to structural surrogates. It can be hypothesized that a favourable evolution of structural parameters contributes, as a BMD-independent determinant, to the previously reported protective effect of E_2 levels on fracture incidence in men (2-5). Of notice, in the cross-sectional analyses, both total E_2 and $BioE_2$ were similarly associated with baseline aBMD and structural parameters, whereas in the longitudinal assessment significant associations were essentially restricted to total E_2. We do not have definitive explanations for this, but as $BioE_2$ is obtained by calculation based on several measurements (i.e. E_2, SHBG, albumine and T), this might imply a greater precision error and in

addition, the longitudinal group is smaller than the cross-sectional study population. In this context, SHBG was not an important determinant of structural bone parameters in this study, possibly because weight is an important determinant of both SHBG and geometric parameters.

In this study, significant correlations of T or BioT with aBMD and morphometric bone parameters are essentially restricted to the univariate cross-sectional analysis. This is in line with earlier data indicating that E_2 is more consistently related to maintenance of aBMD than T in elderly men (12-15). Our results suggest that this is also the case regarding the evolution of HSA assessed morphometric bone parameters in elderly men.

In the cross-sectional multivariate analysis (Table 4a), weight was the strongest determinant for the baseline morphometric HSA variables, except for ED, indicating increasing bone strength by increasing weight. This finding is consistent with Wolff's law (40). Bone mass and structure adapt to physical loading. In non-obese subjects weight is a surrogate for lean and muscle mass which correlates strongly with the forces applied to the bone. For ED height was the most dominant contributor. Weaker independent correlations were provided by TUGT and E_2 levels for aBMD, CSA, avCT and BR. Thus, better physical fitness and higher E2 levels were associated with more favourable femoral neck bone strength surrogates. Increased bone turnover, as assessed by sCTX, weakly, but significantly contributed to higher ED and BR values. This fits with the expectations that increased bone remodeling in the elderly leads to endocortical bone resorption and trabecularisation. In adult men E_2 levels were found to positively modulate the effects of physical activity on a weight bearing bone as the tibia (41). In the present study of elderly males, in the multivariate analysis both E_2 levels and physical performance as assessed by the time up and go test (TUGT) were significant determinants of aBMD and femoral neck structural parameters. However, in the model used the interaction term between these determinants was not significant.

The HSA data could not detect the previously described (42) age-related increase in periosteal diameter (PD) in the cross-sectional analysis (data not shown). Presumably the required sensitivity in this elderly male population with a relatively narrow age span (70-85years) may not have been adequate. During the longitudinal follow-up over 4 years the mean PD increase was modest 0.91% (SD: 2.69%). No significant relationships of PD with any determinant could be found (data not shown). More specifically, T, E_2 nor their bioavailable fractions correlated with PD in the cross-sectional or longitudinal analysis (data not shown).

The structural adaptation to age-related bone loss, e.g. the increase of periosteal perimeter might partially preserve the bending strength under physiologic loads, but cortical thinning (on average by 0.96% over the 4 years follow-up in this study) affects the buckling ratio (+ 2.47% increase / 4year), possibly increasing susceptibility to local buckling failure at the femoral neck in a fall (43).

Low IGF-1 levels have been reported to be associated with decreased BMD in both sexes (44) and found to be involved in the sexual dimorphism in bone geometry occurring during aging (45). However, in the present study neither IGF-1 nor IGF-BP3 were associated with aBMD or HSA derived structural parameters.

CTX showed an independent association with ED and BR in the cross-sectional evaluation (Table 4a) while no correlations of any bone turnover marker with longitudinal assessments of HSA parameters were observed. Intra- and inter-individual variations of the variables are quite substantial and probably leading to lack of power in this cohort to document small effects.

Strength and limitations of the study

The recruitment for this male study was population-based and the study group is quite unique, given the relatively healthy and ambulatory status of men with an age range from 70 to 85 years. Rigorously applied exclusion criteria in both the baseline and longitudinal follow-up groups limit obscuring factors of endogenous sex-steroid effects. A bias towards a selection of the fittest within the longitudinal cohort could have occurred, but the fact that the main baseline characteristics (Table 1) of the longitudinal follow-up population were comparable to these of the overall study cohort argues against. Moreover, drop-out reasons during the longitudinal follow-up are clearly described.

While the geometry assessed by HSA is more sensitive to positioning error the precision in the current study was enhanced by the use of duplicate baseline scans.

The study included both a cross-sectional and a longitudinal evaluation, which can partially reveal relative contributions of determinants to either bone formation and maturation versus age-related bone decline (46).

There are several limitations of the study. The HSA method is not a three-dimensional measurement but is based on two-dimensional DXA technology and relies on several assumptions about shape and trabecular distribution. Differences in rotational positioning between scans reduce precision (47), which are usually greater for geometric parameters compared to aBMD (see methods). Nevertheless, with the exception of

neck width, the bone geometry estimations show expected patterns for age, weight and height.
Femoral neck geometry parameters computed by HSA from DXA data correspond to parameters measured by QCT(48). But clearly, proximal femur breaking strength, estimated by engineering simulations on 2D DXA data is not as well correlated compared to the strength derived by a 3D FEA using QCT data (48).
As bones are complex three dimensional structures, there are inherent limits on the structural information which can be derived from two-dimensional areal projection DXA image data. So, interpretation of the presented relationships of E_2 with the geometrical femoral neck estimates, as contributors to the femoral neck fracture risk, must be done with caution.
The power of this study may be limited for the detection of small changes, weak determinants or interactions between the determinants. There was no fracture endpoint in this study. The prevalence of previous fracture and incidence of new fractures at follow-up were too low for a reliable analysis.

Conclusion
Within the limitations inherent to the applied methodology, the present study provides evidence that higher E_2 levels in elderly men are associated not only with maintenance of BMD but also with more favourable evolution of bone morphometric characteristics, which in turn might contribute to the previously reported association of higher E_2 levels with lower fracture risk in elderly men.

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Table 1. Baseline characteristics of the ambulatory elderly male study population and subset as follow-up.

Parameters	Baseline Population	Follow-up Cohort**
Number	N=269	N=147
Clinical parameters		
Age (year)	76.0 (4.2)	75.0 (3.7)
Weight (kg)	73.4 (11.6)	75.2 (11.5)
Height (cm)	167.7 (6.3)	167.9 (6.2)
BMI (kg/m^2)	26.1 (3.6)	26.6 (3.3)
Smoking (%current/%former)	18.4/59.8	22.2/61.3
Timed Up&Go Test (sec)	10.8 (9.3-13.0)*	10.3 (9.2-12.0)*
Grip strength (kg)	23.5 (8.1)	24.4 (7.7)
Sex steroids		
Total T (ng/dl)	465.9 (148.4)	471.0 (141.4)
Total E_2 (ng/dl)	2.42 (0.68)	2.45 (0.61)
BioT (ng/dl)	166.3 (48.0)	175.1 (44.4)
$BioE_2$ (ng/dl)	1.48 (0.43)	1.54 (0.37)
SHBG (nmol/L)	53.7 (21.1)	50.2 (18.7)
Bone metabolism		
PTH (pg/ml)	45.6 (17.6)	46.1 (17.1)
25OH Vit D (ng/ml)	22.3 (8.8)	23.5 (9.1)
IGF-1 (ng/L)	119.0 (55.9)	125.3 (50.9)
IGF-BP3 (µ/L)	2515 (590)	2592 (582)
OC (µg/L)	14.2 (10.8-18.8)*	13.7 (10.6-17.8)*
BsAP (µg/L)	11.8 (1.0-14.6)*	11.9 (1.0-14.3)*
sCTX (µg/L)	0.366 (0.244-0.494)*	0.347 (0.230-0.451)*

Entries are means (± SD) or medians* (P25-P75) in case of non-gaussian distributions
**Follow–up cohort : all eligible subjects with a 4th year follow-up visit.
T: testosterone, E_2: estradiol, BioT: bioavailable testosterone, $BioE_2$: bioavailable estradiol, SHBG: sex hormone binding globuline, PTH: parathormone, IGF-1 : Insuline growth factor 1; IGF-BP3 : Insuline growth factor binding protein 3; OC: osteocalcin, BsAP: bone specific alkaline phosphatase, sCTX : serum C-terminal telopeptide of type I collagen.

Table 2 : Morphometric estimates of the narrow femoral neck as determined by HAS

	Mean (SD)
Baseline data :	
(n=269)	0.688 (0.126)
areal Bone Mineral Density (aBMD in gr/cm^2)	2.658 (0.620)
Cross-Sectional Area (CSA in cm^2) of bone tissue	1.359 (0.274)
Section Modulus (SM in cm^3)	3.425 (0.272)
Endocortical diameter (ED in cm)	0.130 (0.025)
Average Cortical Thickness (avCT in cm)	15.67 (3.805)
Buckling Ratio (BR : ratio, dimensionless)	
Longitudinal data : (%Δ over 4years from baseline)	
(n=147)	-0.910 (4.875)
%Δ Areal Bone Mineral Density (aBMD)	-0.055 (4.739)
%Δ Cross-Sectional Area (CSA)	0.024 (6.976)
%Δ Section Modulus (SM)	1.075 (3.077)
%Δ Endocortical diameter (ED)	-0.958 (5.113)
%Δ Average Cortical Thickness (avCT)	2.468 (7.602)
%Δ Buckling Ratio (BR)	

Entries are means (± SD); HSA : Hip Structural Analysis (see method section)

Table 3 : Spearman correlations of narrow femoral neck morphometric estimates with sex-steroids and SHBG

	T	Bio T	E_2	Bio E_2	SHBG
Baseline data (N=269):					
areal Bone Mineral Density (aBMD)	0.14*	0.23***	0.21***	0.25***	-0.18**
Cross-Sectional Area (CSA)	0.10	0.20**	0.18**	0.22***	-0.19**
Section Modulus (SM)	0.10	0.15*	0.13*	0.16*	-0.16**
Endocortical diameter (ED)	-0.10	-0.16**	-0.13*	-0.15*	0.03
Average Cortical Thickness (avCT)	0.14*	0.23***	0.21***	0.25***	-0.18**
Buckling Ratio ((BR)	-0.14*	-0.22***	-0.20***	-0.24***	-0.14*
4 year Longitudinal data (N=147):					
%Δ areal Bone Mineral Density (aBMD)	0.14	0.04	0.24**	0.15	0.08
%Δ Cross-Sectional Area (CSA)	0.11	0.01	0.20*	0.12	0.07
%Δ Section Modulus (SM)	0.01	-0.05	0.19*	0.18*	0.01
%Δ Endocortical diameter (ED)	-0.07	-0.06	-0.15	-0.10	-0.06
%Δ Average Cortical Thickness (avCT)	0.13	0.03	0.24**	0.14	0.07
%Δ Buckling Ratio (BR)	-0.12	-0.05	-0.23**	-0.15	-0.07

Entries are correlation coefficients adjusted for age, weight and height
* P<0.05; ** P<0.01; *** P<0.001
T: testosterone , E_2: estradiol, Bio T: bioavailable testosterone, Bio E_2: bioavailable estradiol, SHBG: sex hormone binding globulin

Table 4a: Multivariate analysis of the narrow femoral neck aBMD and morphometric estimates : Cross-sectional data

	aBMD	CSA	SM	ED	avCT	BR
Age	-0.050	-0.025	0.022	0.060	-0.052	0.049
Weight	**0.314*** **	**0.377*** **	**0.361*** **	0.068	**0.311*** **	**-0.222** **
Height	-0.004	0.129	**0.144***	**0.327*** **	-0.007	**0.163** **
T	0.043	0.020	0.035	-0.051	0.042	-0.041
E2	**0.155***	**0.143***	0.119	-0.097	**0.155***	**-0.167***
SHBG	-0.133	-0.101	-0.072	0.111	-0.131	0.126
CTX serum	-0.097	-0.060	-0.007	**0.129***	-0.098	**0.142***
TUGT	**-0.164** **	**-0.141***	-0.117	0.123	**-0.164** **	**0.193** **

Entries are standardized regression coefficients; * P<0.05; ** P<0.01; ***P<0.001
Abbreviations: aBMD: areal Bone Mineral Density, CSA: Cross-Sectional Area, SM: Section Modulus, ED: Endocortical diameter, avCT: Average Cortical Thickness, BR: Buckling Ratio, T: testosterone, E_2: estradiol, SHBG: sex hormone binding protein, sCTX: serum C-terminale telepeptide of collagen type I, TUGT : timed up and go test,

Table 4b: Multivariate analysis of narrow femoral neck aBMD and morphometric estimates : Longitudinal data over 4 years

	%ΔaBMD	**%Δ** CSA	**%Δ** SM	**%Δ** ED	**%Δ** avCT	**%Δ** BR
Age	-0.121	-0.091	-0.094	0.077	-0.128	0.120
Weight	0.136	0.046	-0.045	-0.189	0.134	-0.168
Height	-0.060	0.050	0.035	0.208	-0.061	0.129
T	-0.121	-0.142	**-0.297***	-0.012	-0.134	0.110
E2	**0.241***	0.209	**0.349*** **	-0.097	**0.243***	**-0.242***
SHBG	0.128	0.143	0.134	-0.002	0.129	-0.098
sCTX	0.028	-0.009	0.023	-0.070	0.042	-0.078
TUGT	0.021	0.056	0.158	0.055	0.027	-0.006

Entries are standardized regression coefficients; * P<0.05; ** P<0.01; ***P<0.001
Abbreviation : aBMD: areal Bone Mineral Density, CSA: Cross-Sectional Area, SM: Section Modulus, ED: Endocortical diameter, avCT: Average Cortical Thickness, BR: Buckling Ratio
T: testosterone, E_2: estradiol, SHBG: sex hormone binding protein, sCTX: serum C-terminale telepeptide of collagen type I, TUGT : timed up and go test

Figure 1 :
Quartiles (Q) of E_2 in relation to baseline aBMD and morphometric characteristics at the narrow femoral neck : cross-sectional analysis (n=269). Mean values are represented. Variance analysis was adjusted for age, weight and height. Q1 : < 2 ng/dl; Q2 : 2.0 – 2.2 ng/dl ; Q3 : 2.3 – 2.7 ng/dl; Q4 : ≥ 2.8 ng/dl

Figure 1

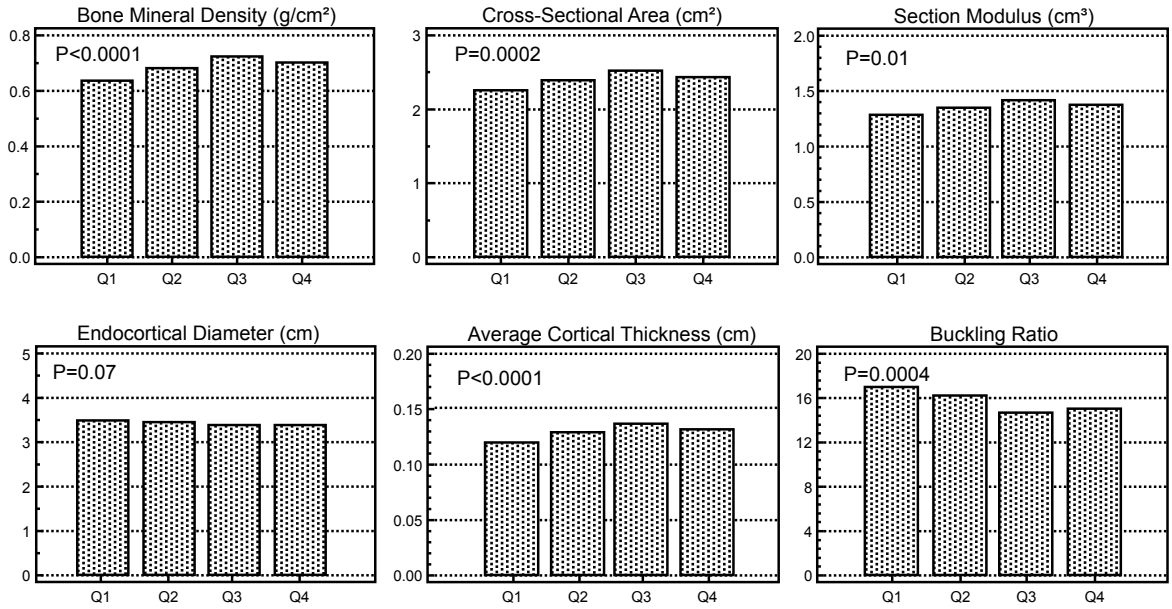
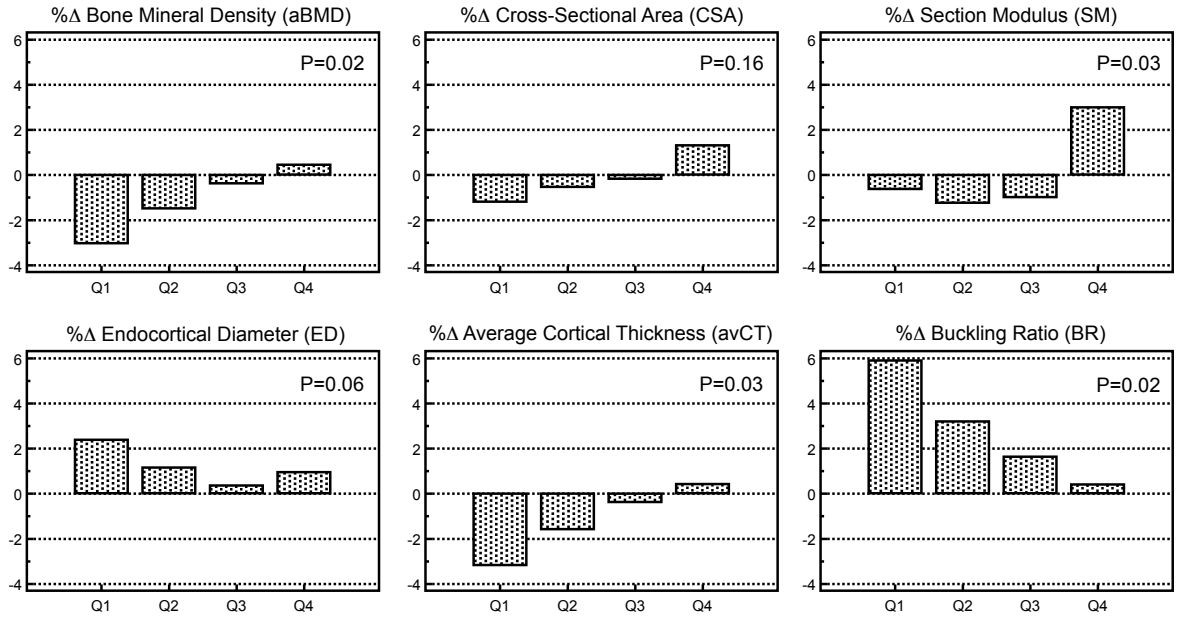


Figure 2 :
Quartiles (Q) of E_2 in relation to %changes of aBMD and morphometric characteristics at the narrow femoral neck over 4 years (n=147). Mean values are represented. Variance analysis was adjusted for age, weight and height. Q1 : < 2 ng/dl; Q2 : 2.0 – 2.2 ng/dl; Q3 : 2.3 – 2.7 ng/dl; Q4 : ≥ 2.8 ng/dl

Figure 2



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Chapter 5

3 Telomere length versus hormonal and bone mineral status in healthy elderly men.

Sofie Bekaert, Inge Van Pottelbergh, Tim De Meyer, Hans Zmierczak, Jean-Marc Kaufman, Piet Van Oostveldt , Stefan Goemaere.

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Chapter 6 General discussion and future perspectives

1 Discussion of the main results

Insights of normal bone biology are important to understand: 1) the development and aging in this particular organ; 2) the consequences of disease and external factors on bone strength as determined by its mass and material and/or structural qualities at all ages and 3) the bone function in the control of mineral homeostasis.
Therefore, the introduction to this thesis aimed at providing an extended summary review of the actual knowledge on bone metabolism as a whole, as a general background for the subsequently discussed clinical bone studies in men.

With the Merelbeke study we wanted to test the hypothesis that the decline of serum testosterone levels in elderly men compared to the levels at the young adult age, are responsible for some clinical aspects of aging in men, whith the skeleton as the primary organ of interest. A main finding of the Merelbeke study is that testosterone changes do not seem to contribute directly to the deterioration of the skeleton in older men and in particular do not appear to play any substantial role in the determination of bone loss. In contrast, the better preserved estradiol (E_2) levels or the only slightly decreased free or bioavailable E_2 fractions are positively contributing to the preservation of skeletal integrity in older men, in particular the maintenance of bone density and femoral neck bone geometry. The results of the Merelbeke study further indicate that in older men a higher bone turnover, as determined by biochemical markers, is a negative determinant of prevalent BMD. There was, however, no indication that between subject variation of sex steroids in elderly men contributes to the determination of bone turnover. Furthermore, although telomere length assessed in peripheral leucocytes was not related to sex steroid levels or prevalent bone density in the older men of the Merelbeke study, telomere length was negatively associated with bone loss at the radius. This suggests a role of aging per se, independently of hormonal changes, in the determination of bone loss in older men. Results discussed in this thesis further allowed to provide a basis to implement a nation-wide, uniform expression of BMD in male patients and allow harmonization of the BMD-based diagnosis and treatment of osteoporosis in men. Finally, results obtained in the Merelbeke study indicated that peripheral bone assessment by DXA at the forearm or heel, or by quantitative ultrasound at the tibia or heel, cannot be used as a substitute for hip DXA, which is commonly considered as the 'reference site' in particular for BMD assessment in older subjects.

2 Contributions of this research to the existing evidence on osteoporosis in men.

2.1 Introduction

2.1.1 Epidemiology of osteoporosis in men

Interestingly, spine and hip fracture incidence rises markedly with aging, but distal radius fracture (Colles' fracture) risk does not change much as men age *(van Staa TP et al, 2001)*. In many regions of western countries, the incidence of hip fracture is stabilizing or decreasing in women, while in men this trend has not been observed or is more modest *(Hopkins RB et al, 2012)*. It is postulated that availability of dual energy X-ray absorptiometry (DXA) and approved treatments of osteoporosis are in part responsible for the decline in hip fractures in women. Men are much less likely to be diagnosed and/or treated for osteoporosis. Until osteoporosis in men is increasingly recognized by both medical professionals and the general public, it is doubtful that the fracture rate will decrease in men.

In general terms, a man at age 50 faces a lifetime fragility fracture risk between 10 and 25 %. Several risk factors and disorders will increase fracture risk, but three major risk groups can be mentioned: 1) men who have already suffered a fragility fracture; 2) men on oral glucocorticoid therapy for at least 3 months and, increasingly; 3) men on androgen deprivation therapy (ADT) for prostate cancer. Men are less likely than women to undergo diagnostic testing or receive treatment after fracture *(Feldstein AC et al, 2005)* despite the fact that men and women have about the same probability of a subsequent fracture *(Center RJ et al, 2007)*. By 3 months of oral glucocorticoid therapy equivalent to about 5 mg of prednisone daily, an increase in fracture risk can be demonstrated *(van Staa TP et al, 2002)*. Men at risk for glucocorticoid-induced osteoporosis are less likely to be diagnosed or treated *(Adler RA & Hochberg MC, 2011)*. ADT is commonly used for patients with prostate cancer. Such men have a good overall survival *(Lu-yao GL et al, 2008)*, but the 5 year fracture risk is as high as 20% *(Shahinian VB et al, 2005)*.

2.1.2 Differential evaluation of fractures in men and women in Belgium

The number of men and women aged 50 years or older amounted to 1.829.000 and 2.130.000 respectively in Belgium in 2010. Data on hip fracture incidence are available for Belgium and the most recent published estimates date from 2005-2007 (Hiligsmann M et al, 2012). The incidence of hip fractures was determined using the national hospital database, which fully covers the annual hospital stays in Belgium. Given that the country specific incidence of vertebral, forearm and, "other" fractures were not found, these were imputed using standardized methods (Kanis JA, et al, 2001c). The incidence of hip fracture (per 100.000 person years) in men and women ≥ 50 years of age was estimated at 228.5 and 538.7 respectively. Differential incidence number by sex and age-class are provided in Table 5.

Table 5 : Incidence per 100.000 person years of hip, clinical vertebral, forearm and "other" fractures in Belgium by age (estimated in 2010 form data in 2005-2007).

Age (years)	Fracture at the Hip	Vertebra	Forearm	Other
		Women		
50-54	27	70	173	179
55-59	53	148	410	469
60-64	84	149	317	324
65-69	140	203	339	477
70-74	271	382	486	768
75-79	606	589	591	1274
80-84	1263	794	791	2148
85+	2371	1115	1012	3983
		Men		
50-54	34	88	32	152
55-59	49	85	74	455
60-64	73	174	137	716
65-69	104	164	157	673
70-74	159	243	102	819
75-79	313	361	89	836
80-84	669	464	130	1778
85+	1371	921	251	3971

Table reproduced from Svedbem A et al, 2013

The number of incident fractures in 2010 was estimated at 80000 (Table 6). Total incident hip, clinical vertebral, forearm and "other" fractures were estimated at 15.000, 12.000, 12.000 and 41.000, respectively; 66% of fractures occured in women.

Table 6 : Estimated number of Incident fractures in Belgium, 2010 .

Age (years)	Hip	Fracture at the Vertebra	Forearm	Other
		Women		
50-74	1.829	2.890	5.562	7.230
75+	8.932	4.676	4.701	16.579
Total	10.761	7.566	10.263	23.809
		Men		
50-74	1.280	2.293	1.490	8.571
75+	2.919	2.187	605	8.148
Total	4.199	4.480	2.095	16.719

Table reproduced from Svedbem A et al, 2013

A prior fracture was defined as a fracture in an individual who was alive during the index year (2010) which had occured after the age of 50 years and before 2010. In the population over 50 years of age, the number of individuals with hip and vertebral fractures that occured before 2010 was estimated at 74.000 and 81.000 respectively.

The incidence of causally related deaths (per 100.000 in age class) in the first year after fracture comprises approximately 30% of deaths associated with fracture (Kanis JA et al, 2003). The mumber of causally related deaths in 2010 was estimated at 979. Hip, vertebral and "other fractures accounted for 492, 310, and 177 deaths respectively. Overall, approximately 51% of deaths occurred in women.

The cost of a hip fracture has been estimated at € 11.426 in Belgium (Bouee S et al, 2006).
Given that no cost data for the other fractures were available, these were imputed by standard methods (Kanis JA et al, 2001). Longterm disability costs were estimated by multiplying the yearly cost of residing in nursing home (Authier P et al, 2000) with the simulated number of individuals with prior fractures that had been transferred to nursing homes due to the fracture.
The costs of osteoporosis in 2010 was estimated at € 606 million. First year costs and subsequent year costs and pharmacological fracture prevention costs amounted to € 419 million, € 157 million and € 29 million, respectively. It is notable that the pharmacological fracture prevention costs amounted to only 4.8% of the total costs. When stratifying cost of osteoporosis by fracture type, hip fractures were most costly (€ 308 million), followed by "other" (€ 232 million), spine (€ 28 million) and forearm fractures (€ 9 million).

The number of quality adjusted life years (QALYs) lost due to osteoporosis in 2010 was estimated at 26.800; 67% of the total QALY loss was incurred in women. Prior fractures accounted for 55% of the total QALY loss. When the cost of osteoporosis was combined with the value for QALYs lost (valued at 2 x gross domestic product (GDP)), the cost of osteoporosis mounted to € 2.34 billion in Belgium in 2010. Incident fracture, prior fracture, pharmacological fracture prevention, and value QALYs lost accounted for 18%, 7%, 1% and 74% respectively.

The population above 50 years of age is expected to increase from 4.0 in 2010 to 4.6 million in 2025, corresponding to an increase of 17%. The total number of fractures is estimated to rise from 80.000 to 99.000 in 2025, corresponding to an increase of 24%. This increase is estimated to be particularly marked in men (+32%) compared to women (+20%); when no change in the age and sex specific incidence was assumed over this period.
The cost of osteoporosis (excluding the values of QALYs lost) was estimated to rise from € 606 million in 2010 to € 733 million in 2015, corresponding to an increase of 21%. The cost incurred in women and men increased by 17% and 29% respectively.
The total number of QALYs lost due to fracture was estimated to rise from 26.800 in 31.300 in 2025, corresponding to and increase of 17%. The increase was estimated to be particularly in men (+25%) compared to women (+13%).
The cost of osteoporosis including value of QALYs lost was estimated to increase from approximately € 2.3 billion in 2010 to €2.8 billion in 2025. The increase was estimated to be particularly marked in men (+26%) compared to women (+14%).

The proportion of persons over the age of 50 years who were treated for osteoporosis increased from 2% in 2001 to 6.3% in 2011, and there after decreased.

The treatment gaps in men and women were estimated at 45% and 47% respectively. Note that these estimates were conservative, given that it assumes that current use of osteoporosis treatments are only directed to men and women at high risk. Not all individuals at high risk as assessed by e.g. FRAX are eligibel for reimbursement with the present reimbursement criteria.

2.1.3 Pathophysiology of osteoporosis in men

The higher fracture risk in women compared to men is, besides to their lower total bone mass and smaller bone size at the young adult age, partially related to the dramatic loss of estrogen with the cessation of menses. This acute estrogen deficiency leads to a rapid and excessive deterioration of the trabecular and cortical bone compartment in terms of loss of mass and structure.

The results presented in this thesis were derived from a cross-sectional study and longitudinal follow-up of data collected from a cohort of community-dwelling older males in a well-defined community in the late nineties. Globally, our results were in agreement with those of other studies at that time and later, confirmed and extended new insights in the relationships between sex steroid hormones and bone characteristics.

In men, there is no dramatic decrease in sex steroid secretion, analogous to the radical changes during menopausal transition in women. Studies in older men have demonstrated that serum estradiol levels, mostly derived from aromatization of testosterone, are inversely and more closely related to osteoporosis indicators like bone density and fracture than testosterone. A detailed description of the separate roles estradiol and testosterone is provided in Endocrine Reviews *(Kaufman JM & Vermeulen A, 2005)* and more recently in Osteoporosis International *(Merlotti D et al, 2011).*

The relation between bone health and sex steroids in men is complex. In animal models and in humans, there is evidence for direct effects on bone by both androgens and estrogens *(Wiren KM et al, 2002; Lee H et al, 2006)*. Among the many potentially important factors is the evidence for a threshold level of E_2 needed for skeletal maintenance in men. As said, in men estradiol is mainly derived via aromatization of testosterone. There are both genetic and environmental effects on aromatase activity, most dramatically illustrated by the continued long bone growth, low bone density, and lack of epiphyseal closure in young men lacking aromatase. Aromatase is found in many cells, including osteoblasts and adipocytes, and may be affected by medications and environmental endocrine disruptors. Greater understanding of aromatase regulation may lead to newer therapeutic approaches to osteoporosis in men.

One area of great interest has been whether the apparent decreases in serum testosterone (T) and free testosterone (FT) with aging have any impact on osteoporosis and fracture risk. Several cross-sectional and longitudinal studies *(Harman SM et al, 2001, Kaufman JM & Vermeulen A, 2005)* have demonstrated decreases in serum total and free testosterone that seem to parallel the gradual decrease in bone mineral density with aging. Is there any connection? Two findings make this unlikely.

First, in older men, bone mineral density is much more closely related to serum bioavailable estradiol than any measure of androgen *(Khosla S et al, 2001b)* although the major source of estradiol in men is aromatization of testosterone.

Second, it has been questioned whether age-related decline of T results from aging itself of from illness associated with aging *(Sartorius S et al, 2012)*. An important example of this is the secondary hypogonadism found commonly in obese, diabetic men *(Dandona P & Dhindsa S, 2011)*. For many years, it has been postulated that significant chronic disease was considered a risk factor for osteoporosis in men. Chronic disease-induced hypogonadism could contribute to fracture risk by lowering testosterone secretion, providing less substrate for conversion to estradiol and perhaps by affecting lower body muscle strength, leading to falls *(Orwoll ES et al, 2006)*. This requires further study.

Type 2 diabetes (T2D) is associated with increased fracture risk (reported Odd Ratios are around 1.5). Unlike osteoporosis, diabetic fractures are associated with obesity and normal to high bone mineral density. Those two factors are typically associated with reduced fracture risk. It is consequently suggested that the increased fracture risk in T2D results from abnormalities in bone material strength (BMS) and / or bone architecture (bone "quality"). Additional studies are needed to characterize bone material strength and microstructure in T2D, particularly in men and in nonwhite populations. Impaired skeletal load response (stress) exists in women with T2D (Hamilton CJ et al, 2013). This could contribute to an increased fracture risk and sheds light on the so-called T2D and BMD paradox.

Other endocrine causes of secondary osteoporosis include hyperparathyroidism, hyperthyroidism, and endogenous glucocorticoid excess (Cushing's syndrome). There are many gastrointestinal disorders that are associated with osteoporosis, including celiac disease (which may be relatively asymptomatic), inflammatory bowel disease, and exocrine pancreatic deficiency. Iatrogenic causes include the now no longer performed surgery for peptic ulcer disease, such as the Billroth procedures and more recently bariatric surgery. Increased number of surgical procedures for obesity are likely to produce many patients at risk for fracture. However, obesity itself may be a risk factor for fracture in men. In the MrOS study *(Nielson CM et al, 2011)* of about 6,000 US men, men with BMIs of 30–34.9 kg/m^2 had a 29 % relative increased risk of non-spine fracture. For men with BMIs of 35–39.9 kg/m^2, the relative risk was almost double that of normal weight men. Some of the association was due to poorer physical functioning in obese men.

Other forms of secondary osteoporosis in men can be documented by more extensive laboratory evaluation and specific clinical causes of osteoporosis are of particular importance and were extensively reviewed *(Ryan R et al, 2011, Fitzpatrick AL, 2002, Adler RA, 2012, Mazziotti G et al, 2010)*. In the Swedish arm of the MrOs study it was found that a high hsCRP was associated, independently of BMD, with an increased fracture risk, and in particular an increased vertebral fracture risk *(Eriksson et al 2014)*.

Important causes of secondary osteoporosis in men in addition to the two secondary causes already mentioned (glucocorticoids and ADT) are: 1) hypercalciuria *(Ryan LE & Ing SW, 2012)* by which some of these men may present with nephrolithiasis, while other cases will be discovered during the evaluation of osteoporosis; 2) other forms of secondary hypogonadism other than that due to ADT, as e.g. hypogonadism from alcohol abuse which may contribute to the increased fracture risk found in men. A long list of medications (in addition to glucocorticoids and ADT) has been associated with increased fracture risk, such as proton pump inhibitors, thiazolidinediones, anti-depressants, anti-seizure medications, neuroleptics, immunosuppressives and cancer chemotherapy drugs *(Mazziotti G et al, 2010)*.

Risk factors for hip and other major osteoporotic fractures (spine, forearm, humerus) have been identified by epidemiological studies and are used to construct fracture risk calculators, such as FRAX *(Kanis JA et al, 2010)* and the Garvan nomogram *(Nguyen ND et al, 2008)*. These risk factors may provide hints to the pathophysiology, but they do not truly provide mechanistic information.

2.1.4 Evaluation of osteoporosis in men

It is obvious that medical history and physical examination should reveal information about risk factors and potential secondary causes. In a review *(Ryan CR et al, 2011)* of men referred to an osteoporosis clinic, the great majority of the men had multiple risk factors and potential secondary causes. Laboratory testing *(Adler RA, 2012)* should include serum chemistry (calcium, phosphate, creatinine, albumin), a complete blood count and protein electrophoresis (as a clue to multiple myeloma, which can cause pathologic fractures), a serum 25-hydroxyvitamin D level and PTH) and a 24-hour urine for calcium and creatinine.

Hypogonadism should be excluded, at least if risk factors or suspicious symptoms are apparent, by measurement of testosterone and sex hormone binding globulin and calculated free or bioavailable forms. In a second stage in case of confirmed hypogonadism luteinizing hormone (LH) follicle stimulating hormone (FSH) and prolactine should be considered. For older men with osteoporosis and/or fragility fracture, one may first ask if the patient is a candidate for testosterone replacement, should a low serum

testosterone be discovered. The role of testosterone as a treatment for osteoporosis is discussed below and is important in the decision about whether serum testosterone testing is indicated.

Men with hypercalcemia or hypocalcemia will also need measurement of parathyroid hormone (PTH). Normocalcemic hyperparathyroidism *(Bilezikian JP & Silverberg SJ, 2010)* may present with low bone mass; some experts suggest that all patients with osteoporosis should have a serum PTH level assessed, regardless of serum calcium level.

DXA of the hip and spine remains the gold standard test for osteoporosis. In those men in whom either the spine or hip cannot be measured accurately (usually because of degenerative changes), a forearm bone density is recommended, usually the distal 1/3 radius *(Binkley N & Adler RA, 2010)*. In addition, forearm bone density is often helpful for assessing patients with preferable cortical bone loss as e.g. hyperparathyroidism, hyperthyroidism and prostate cancer on ADT. As in our studies of healthy men (also outside the Merelbeke study) mean Z-scores (based on Hologic references) in the healthy control subjects were at -0.4 to -0.5 at all forearm subregions, for Belgian men the use of the Hologic forearm reference database will result in an overdiagnosis of osteoporosis.

Osteoporosis was defined by the World Health Organization *(Kanis JA et al, 1994)* as a spine or hip bone density 2.5 standard deviations below the mean bone density of young healthy adults at the age of peak bone mass. This definition was developed for Caucasian women, but the terms have been used for other female populations and for men. The FRAX calculation uses the absolute bone mineral density in g/cm^2 because some studies *(Kanis JA et al, 2011)* show that men and women fracture at about the same bone density. However, in other studies *(Selby PL et al, 2000)* men with fractures had a higher absolute bone mineral density.

Hence some experts *(Adler RA & Hochberg MC, 2011a)* have argued that men should be compared with men and that a male normative database should be used. The larger bones of men also make the bone density appear better than it is *(Binkley N et al, 2010)*. Probably the most compelling reason to use the male database is that those men who have osteoporosis based on this database or who have low bone mass (osteopenia defined as a T score between -1 and -2.5) plus a previous fragility fracture, responded to standard therapy in the various trials in men. Using the female database will exclude some of the men who would have responded to treatment.

2.1.5 Treatment of osteoporosis in men

While treatment of an underlying secondary cause of osteoporosis may improve bone mineral density and fracture risk, many men with secondary and those with primary osteoporosis will require specific osteoporosis treatment. Many of the medications that have been used to treat osteoporosis in women have been tested afterwards in men. In general, these agents increase bone mineral density and change bone turnover markers similarly in women and men. It is important to note that the registration trials for such drugs have been done in women and sample sizes were large enough to demonstrate that the medications lead to fewer clinical fractures. Up until now, studies in men have mostly not had sample sizes large enough to show sex-specific decreases in fracture risk.

As stated before, osteoporosis has only recently been appreciated as a major medical problem in men. Methods for evaluation and treatment have been reviewed by the author of this thesis *(Kaufman JM & Goemaere S, 2008)* and others *(Boonen S, 2007, Khosla S, 2010,Kaufman JM, 2013)*. More recently a tasks force of the Endocrine Society has developed evidence-based practice guidelines *(Watts NG et al, 2012)* on diagnosis, evaluation, and management of osteoporosis in men. Nonetheless, while there are many unanswered questions about present management, recognition of the problem and its consequences remains low. This is particularly important because osteoporotic hip fracture is often a fatal disorder in older men. After hip fracture, men are about twice as likely to die than women *(Block JE & Stubbs H, 1997)*. Those that survive are more likely to loose independence. Thus, recognition of the problem, efficient evaluation, and prompt treatment will likely lead not only to fewer fractures but also to fewer fracture-related deaths.

Recommendation for regulatory requirements for registration of drugs intended for use in the treatment in male osteoporosis have been provided many years ago (Kaufman JM et al, 1999, Reginster JY et al, 2006). It is recommended that an indication could be granted for the treatment of osteoporosis in males on the basis of a placebo-controlled study, with bone mineral density changes after 1 year as the primary endpoint, for indications approved in the treatment of postmenopausal women at high risk of fractures.

Recently, a report on treatment of osteoporosis in men from a panel debate of experts in the European Society for Clincial and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) was published (Kaufman JM et al, 2013). The content of this report is summarized here.
Awareness in osteoporosis in men is improving, although it remains under-diagnosed and under-treated. Although additional fracture data are needed to endorse the clinical care of osteoporosis in men, consensus views were reached on diagnostic criteria and intervention thresholds. Consensus views on the BMD diagnosis of osteoporosis in men included the use of DXA technology, femoral neck as the reference site and the data from the NHANES III for women aged 20-29 years as basis to calculate T-scores (Kanis et al, 2011).

In terms of assessment algorithms, different approaches have been used, either the traditional approach or a fracture probability-based approach, as is the case in the UK. FRAX is, however, increasingly used in guidelines as they undergo revision.

The treatment algorithm and clinical guidance aim to treat men at a similar 10-year fracture risk as in women, because the morbidity and mortality associated with major osteoporotic fractures in men are substantial. Available evidence suggests that treatment algorithms in women are also applicable to men. In practice, this is likely to involve FRAX and clinical risk factors. The use of fixed intervention thresholds is viewed as counter-intuitive to current practice, because the risk is to exclude too many younger patients, and conversely, to include too many older patients above a fixed threshold value.

The available level of evidence that treatment decreases the risk of fracture in men is lower than for women. As such, the US Endocrine Society is of the opinion, that there is currently not enough information in men to make a recommendation, because too few fractures have been recorded to link BMD changes with anti-fracture efficacy. However, the ESCEO panel (Kaufman JM et al, 2013) believes that this view can be countered, based on the available epidemiological and clinical efficacy data in male subjects, which display similarities with data acquired in women, in terms of treatment effects of BMD, biochemical markers of bone turnover, and fracture endpoints, despite the recorded differences in pathophysiology of bone loss and bone architecture. Overal, empirical data from men and women are so similar that differences in morphology may not be clinically relevant.

2.1.6 Evalation of the bone density and osteoporosis in the Merelbeke Study

2.1.6.1 General aspects

Bone densitometry by dual-energy X-ray absorptiometry (DXA) is the standard technology by which the diagnosis of osteoporosis is made. In 1994 a DXA-based definition using T-scores (standard deviation differences with BMD values at the peak bone age) was proposed in a WHO technical report *(Kanis JA et al, 1994)*. By that time this operational definition was only validated for postmenopausal women for BMD data at the lumbar spine, proximal femur and distal forearm. The choice of the T-score of -2.5 was based on epidemiological considerations to identify an approximate of one third of the postmenopausal female population. This cutoff was chosen to reflect the estimated prevalence of fragility fractures in one of three postmenopausal women. Later prospective data on the value of baseline DXA values in women and thereafter in men in predicting fragility fractures became available *(De Laet C et al, 1998)*. It was illustrated that in men exactly the same relations are operational as in postmenopausal women.

Although fracture prediction is most optimal by site-specific measurements, a general mean rule states that by every standard deviation (SD) decrease of BMD the fracture risk doubles.

There is no general consensus on a densitometric definition of male osteoporosis *(Kanis J et al, 2011)*. Discussion points on the standardisation of this aspect were the reference population used and the threshold. The NHANES III reference *(Looker AC et al, 1995)* is accepted most broadly worldwide. The best absolute fracture prediction of the hip was provided by the same absolute femoral neck BMD values used to diagnose densitometric osteoporosis in postmenopausal women. This means that for diagnosing osteoporosis in men a T-score cut-off is used comparing the male absolute values with a female reference database.

2.1.6.2 Comparison between bone evaluation techniques

DXA based BMD at the proximal femur is a standard reference for the diagnosis of osteoporosis and an important factor for the calculation of the absolute fracture risk in women and men.
As was found for females, bone evaluations by DXA or quantitative ultrasound (QUS) at peripheral sites (forearm, tibia or heel) in the older men of the Merelbeke Study population performed only moderately in predicting standard DXA based central (e.g. at the hip) BMD. Correlations and weighted Kappa analyses revealed only weak associations and concordance, respectively, between non-site-specific and technique-specific measurements. These discrepancies in absolute values result from a variety of factors. These can be site-specific processes during skeletal development and/or aging, and/or technical issues such as accuracy biases in data acquisition and/or impact of bone geometry according to the bone site and/or measurement technique and/or patient positioning (e.g degree of endorotation).
As a result, in the Merelbeke Study population, as well as in other studies *(Szulc P et al, 2000)* substantial discrepancies remained when T-scores were used for the calculation of prevalences of osteoporosis between different measurements.

Using the practical and user-friendly peripheral measurement devices (less expensive, mobile, low or no irradiaton) to predict a "gold standard" such as the hip BMD, reveal by ROC analysis that all sites and applied technologies (except the speed of sound (SOS) at the midtibia) performed equally well. However, the predictive power is too low to use these measurements as an alternative for hip BMD for diagnostic purposes (AUC <0.800). In this regard, the data in the Merelbeke Study are in accordance with other findings *(Adler R et al, 2001)*.

From a prescreening point of view, the use of cheaper and more mobile peripheral measurement devices (heel QUS or BMD or distal forearm BMD) at a cuttoff with a diagnostic sensitivity of 90% could allow to reduce the referrals to DXA by 35 to 50%. The cost-effectiveness of such a strategy should be studied in a clinical setting.
Peripheral bone measurements were investigated in studies on fracture prediction in men *(Gärdsell P et al, 1990, Ross PD et al, 1999)*. The prospectively assessed predictive value of peripheral measurement, is in accordance with the capacity of peripheral BMD and QUS techniques to discriminate non-traumatic fracture patients from non-fracture matched controls groups. These observations were confirmed in the cross-sectional analyses of the Merelbeke Study population.

Based on the available literature the value of quantitative ultrasound (QUS) in osteoporosis management is similar in men and women. The clinical utilisation of heel QUS depends on its ability to predict clinical fractures. This is particularly important in settings with no or very limited access to DXA.
In a meta-analysis (Moayyeri A et al, 2012) including 21 prospective heel QUS studies (with 55.164 women and 13.742 men with a follow-up of 279.124 person-years) the predictive power for fracture was estimated. The relative risk (RR) per standard deviation (SD) of different QUS parameters (broadband ultrasound attenuation (BUA), speed of sound (SOS), stiffness index (SI) and quantitative ultrasound index (QUI)) for various fracture outcomes (hip, vertebral, any clinical, any osteoporotic and major osteoporotic fractures) were reported. All four QUS parameters were associated with risk of different fracture. RR for hip fracture for 1 SD decrease in BUA was 1,69 (95% CI 1.43-2.00), SOS was 1.96 (95% CI 1.64-2.34), SI was 2.26 (95% CI 1.71-2.99) and QUI was 1.99 (95% CI 1.49-2.67). Validated devices from different manufacturers predicted fracture with similar performance. QUS measures predicted fracture with a

similar performance in men and women. The QUS measures adjusted for hip BMD showed a significant and independent association with fracture risk (RR/SD for BUA = 1.34 (95% CI 1.22-1.49)). Further research is needed for more widespread utilisation of the heel QUS in clinical settings. The added value of QUS should be weighted against other clinical risk factor (e.g. those included in the FRAX algorithm). Indeed the reproducibility of the standardized and validated QUS devices (at least %CVs of 3 to 6%) is much less than that for DXA-BMD (usually with % CVs between 1 and 2%). Consequently, knowing the responses to treatments, the signal to noise value does not indicate QUS as a good technique to monitor patient's osteoporosis therapy.
The main problems of QUS measures are that it is not clear which physical characteristic is measured, the inferior standardization and inferior device stability compared to DXA.

In respect to the longitudinal subject/patient follow-up, the Merelbeke study confirms that compared to DXA, QUS techniques are at least twice less reproducible. This precludes a preference choice for QUS in longitudinal bone loss or bone gain studies. Consequently, the QUS measurements were not maintained in the follow-up in the Merelbeke Study population.

2.1.6.3 Standardization of DXA BMD evaluation

Bone mass measurements are usually expressed in respect to a reference population. T- or Z-score are the most widely accepted terms. A T-score compares the subject/patient value with the mean value at the young adult age. A Z-score compares this with a age- and sex-matched mean. The scores are usually expressed in standard deviations and less frequently by precentages.
There is a great interest in standardizing absolute BMD values, to allow formal comparison between values obtained on devices from different manufacturers. Lacking standardization has resulted in differences in reported young normal values and SD scores for dual X-ray absorptiometry devices from different manufacturers, i.e. for the devices from the Hologic, Lunar and Norland companies.
Although formulae for the conversion of manufacturer-derived absolute BMD values (expressed in mg/cm^2) were developped in female populations, using these formulae to calculate standardized BMD (sBMD) values in men resulted in the reduction of the intersite-variability in European Standard Phantom (ESP) to less than 2%.
European studies using local reference values have reported important difference in the prevalence of osteopenia and osteoporosis as compared to the use NHANES III from US. To provide a uniform basis for the diagnosis of male osteoporosis, a national male reference sample was established as a collaborative effort between major osteoporosis centers in Belgium. A single, nation-wide reference sample avoids the inconsistencies that result from the use of different, manufacturer-derived reference poplations.

The findings in the Belgian young adult male reference population demonstrated similar peak BMD and SD compared to the US population, both on Hologic and Lunar. The only exception was the Hologic lumbar spine BMD. The lower standard BMD (sBMD) values at the L1/L2-L4 on the Belgian fan-beam Hologic densitometers compared to those obtained on older US pencil- beam Hologic devices, are however most probably due to true population differences. In the Hologic database the reference population for the spine and proximal femur were different and fan-beam vs pencil-beam cross-calibration studies usually report differences less than 2%. The pertaining small difference (1.6 to 2.1%) between the global Belgian and US device independent sBMD mean values are considered to be of no clinical relevance.

The standardization efforts in women and in the presently reported Belgian male study are important in respect to generate a uniform framework for the diagnosis and treatment of osteoporosis. By calculating sBMD values and by providing national reference values, osteoporosis can be uniformally diagnosed across different centers using different devices.
The T-score concept has been initially accepted for a diagnostic categorisation and later on proposed for treatment guidelines and/or reimbursement criteria. In the early years of this century it has been documented that antiresorptive drugs and teriparatide are likely to reduce fracture risk to a similar extent in both sexes.

All the previous work of standardization in Belgium, leading to a harmonisation of the DXA-based diagnosis of osteoporosis and drug therapy in women and men with osteoporosis, has been performed in collaboration with the manufacturers of the DXA devices. The Belgian affiliates of Hologic and Lunar implemented the calculation of sBMD values in their report in addition to the devices specific absolute BMD values.

However, these indices were not taken into account in the Belgian reimbursement criteria, potentially leading to inadequate treatment in a fraction of patients.
All the previous work on standardization in Belgium might be meaningless in the absence of a valid quality control system (as e.g. is implemented in France). As Belgium is the country with the highest prevalence of DXA devices with respect to its 11 million inhabitans, the question is how these instruments are used in clinical practice.

2.2 Bone turnover in older men

Biochemical indices of bone turnover can easily be assessed by a variety of standardized tests either in serum or urine samples. In the Merelbeke Study the assessed bone turnover indices of older community-dwelling males were as expected compared to other studies. The older men values were lower than young adult male controls and compared to the middle age reference in the study (sons of the older study subjects) a rather limited and non-consistent increase of bone turnover markers was observed. Bone turnover parameters were negatively associated with areal BMD in the male study subjects, comparable to such relationships in female. These findings contrast with the absence of correlations between male bone turover and BMD in younger men, providing arguments that increase of bone bone turnover favors aging bone loss and suggesting that antiresorptive drugs might be usefull for its prevention.

As a confirmation of previous studies, in the Merelbeke Study approximately 50% lower free testosterone (FT) and 70% lower DHEAS were found as compared to the young reference group. Also, the relatively well-preserved total E_2 levels were in agreement with previous data and reflect that the decreased substrate availability, is compensated for by an increased aromatisation of T. The 12% decrease observed in free E_2 (FE_2) resulting from an increase of sex hormone binding globulin (SHBG) was smaller than previously reported in other studies.

It was demonstrated that bone turnover parameters in the older men were at best marginally affected by differences in sex steroid levels both in univariate and multivariate analyses. Total T was not correlated. The observed negative correlations with FT, E_2 and FE_2 were weak and inconsistent.

Several reasons for the apparent limited influence of the relative sex steroid deficiency on bone turnover indices could be proposed: 1) the FT and/or FE_2 in the healthy men were above a threshold below which bone turnover increases more markedly; 2) interfering factors (e.g. age, BMI, lifestyle, PTH, vitamin D, kidney function and others …) could have obscured a relationship; however, additional multivariate analyses did not change the relationships; 3) the relationships are to weak to be more consistently demonstrated by this cross-sectional sample of the Merelbeke Study.
These older male data do not support that the observed positive correlations between sex steroid levels and BMD, are mediated primarily by bone turnover change.

Although, such a pathophysiologic pathway could have been missed by the merely marginal effects of partial sex steroid deficiency cumulated over long time or by large precision errors in the assessment of bone turnover markers. The alternative explanation could be that positive cross-sectional correlation of T and E_2 with BMD are in part residual effects of the sex steroids on bone earlier in live, although our longitudinal data do indicate a role of sex steroids also in bone loss.

2.3 Bone loss in older men

2.3.1 Effect of sex steroids on bone mineral density changes

In community-dwelling older men in Merelbeke, bioavailable estradiol ($bioE_2$) was consistently associated with prospectively assessed BMD changes at all measured sites. Higher E_2 levels was followed with smaller BMD losses during the longitudinal follow-up. The *CYP19* $(TTTA)_n$-repeat polymorphism was, moreover, an additional independent determinant of BMD changes at the distal forearm. Furthermore, in this population of ambulatory older men, the *CYP19* genotype was associated with self-reported clinical fracture risk.

The observed consistent association in the Merelbeke older male cohort between serum $bioE_2$ levels and prevalent BMD assessed both at the forearm and at the proximal femur is in agreement with several previous reports in aging men. However, cross-sectional associations between $BioE_2$ and BMD do not allow the establishment of a role for $bioE_2$ in age-associated bone loss. In this context, we found only marginal associations between biochemical markers of bone resorption and prevalent $bioE_2$ in the Merelbeke Study analyses. Nevertheless, in some other studies in older men, an inverse association between biochemical markers of bone turnover and $BioE_2$ was more clearly observed.

Other studies have prospectively assessed BMD changes in older men. In a population based cohort from Rochester an association was observed between $BioE_2$ and BMD changes at the mid-region of the forearm in men aged over 60 year *(Khosla S et al, 2001b)*. In the Osteoporotic Fracture in Men Study low $bioE_2$ and high SHBG were associated with lower BMD and faster hip BMD loss *(Cauley JA et al, 2010)*.

The Merelbeke Study, which concerns a sizeable and homogenous group of ambulatory men over 70 years of age at initiation of the study, allowed us to confirm and extend previous finding by revealing a consistent association between $bioE_2$ and %BMD changes at different subregions of the forearm as well as at the proximal femur.

From the results of the Merelbeke Study it appears that the correlation between the E_2 levels and BMD or hip geometry estimates (or their changes over time) seem to follow a linear track without clear indications for a threshold effect, although in some analyses a more marked (non-significant) difference was observed for the lowest E_2 quartile.
Some studies indeed seem to confirm those findings (Szulc P et al, 2001, Cauley J et al, 2010).
In contrast to our findings in the Merelbeke study, several studies in other populations, with a broader age range did suggest threshold E_2 values, below which men begin to loose bone either at radius, spine or femoral neck (Khosla S et al, 2001b, Genarri L et al, 2003). In the age-stratified population studies at the Mayo Clinic (Rochester, Minisota), in which serum bioavailable estradiol ($BioE_2$) continuously declined as a funtion of age in men (Khosla S et al, 1998), those findings were further elaborated using central and peripheral quantitative computed tomography (QCT) (Camp JJ et al, 2004; Riggs BL et al, 2004). The presumed threshold in men was most evident for cortical bone (Khosla S et al, 2005). At all cortical sites assessed in men, volumetric density (vBMD) was associated with serum $bioE_2$ level at low (< 8 pg/ml (30 pmol/L)), but not at high (≥ 8 pg/ml) levels. No such differences were evident at trabecular sites, where the associations between $bioE_2$ and trabecular vBMD were similar at low versus high $bioE_2$ levels. These data suggest that the threshold for estrogen deficiency in cortical bone is considerably lower than that in trabebular bone. For the MrOs Sweden study cohort a non-linear inverse relation between serum (free)E_2 and fracture risk was reported, with a strong relationship below 16pg/mL E2 or 0,3pg/mL freeE_2 (Mellström et al 2008).
These constrasting data with those from the Merelbeke study might be due the narrower age range of the male population in the Merebeke study, the lower number of study subjects and/or differences in the E_2 analysis methods. Nevertheless, more linear patterns of inverse association of E2 with adverse bone indices such as bone loss have also been reported for large cohorts of elderly men and E2 measurements with state of the art mass spectrometry-based assays, such as in the US MrOS cohort (Cauley et al 2010).
In any case whether or not there is a threshold effect, all studies including the Merelbeke study concurr to indicate that the lowest free- or $bioE_2$ levels, i.e. in the lowest quartile, are consistently associated with adverse skeletal effects.

Estrogen biosynthesis necessarily involves the aromatase enzyme, which is expressed in a variety of tissues. The hypothesis that the functional *CYP19* $(TTTA)_n$-repeat polymorphism may quantitatively

modulate estrogenic activity on bone metabolism was explored. In the Merelbeke Study with E_2 determined by immunoassay, no relationship between the *CYP19* genotype and circulating levels of $bioE_2$, bioT or LH were found, nor with the T/E_2 ratio. The power calculation of the Merelbeke Study indicated a 80% power to detect a 10% difference in hormone levels. An association between the *CYP19* $(TTTA)_n$-repeat polymorphism and circulating E_2 levels was previously reported in women (23). A similar association was also reported in young and older men *(Eriksson AL et al, 2009, Gennari L et al, 2004)*.

Direct association between the *CYP19* $(TTTA)_n$-repeat polymorphism and bone mineral metabolism were explored. Whereas there was no correlation between the *CYP19* genotype and prevalent BMD, the *CYP19* genotype was associated with %BMD change at the distal forearm in both univariate and multivariate analysis. Interestingly, in multivariate models, the *CYP19* genotype was found to affect %BMD change independently of circulating $BioE_2$ levels, thus suggesting a possible local effect of the *CYP19* genotype at the bone tissue level. Hence, estrogen activity at the site of action in bone may not be correctly reflected in the measured circulating $bioE_2$ levels *(Simpson ER & Davis SR, 2011)*. In this context, it is interesting to note that a tissue-specific regulation of aromatase enzyme expression has been reported. Moreover, studies focusing on modulating effects of *CYP19* polymorphisms on breast cancer risk, observed an association of the repeat length TT genotype and e.g. the breast cancer tumor size.

Functional effects of TT genotype and a longer *CYP19* $(TTTA)_n$- repeat length can be hypothesized. In respect of age-related bone loss longer *CYP19* $(TTTA)_n$-repeat length would then be associated with a high aromatase activity phenotype and less pronounced age-related bone loss. Despite the *in vitro* data on a potential functional impact of the assessed *CYP19* gene polymorphism, the possibility that the polymorphism is in linkage disequilibrium with other not yet identified genes remains.

There have been associations between the *CYP19* $(TTTA)_n$-repeat polymorphism and cross-sectional BMD at the spine in postmenopausal women *(Masi L et al, 2001)* and with rates of bone loss at the spine in older men have been reported *(Gennari L et al, 2004)*.

Only 8.4% of the Merelbeke study subjects reported having sustained a fracture after the age of 50. There was no significant association between bioE levels and clinical fractures. However, the *CYP19* genotype was associated with clinical fracture history in our study subjects.

In the Rancho Bernardo study, a relationship between $bioE_2$ levels and radiologically defined vertebral fractures in older men was observed *(Barrett-Connor E et al, 2000)*. A relationship of low serum E_2 and high SHBG with fracture incidence was subsequently reported for the MrOS Sweden Study (Mellström D et al, 2008). In an Italian population a higher incidence of radiologically assessed vertebral fractures in postmenopausal women with shorter *CYP19* $(TTTA)_n$-repeat alleles *(Masi L 2001)*.

As an association study does not prove causality, there are some speculations about the role of the *CYP19* $(TTTA)_n$-repeat polymorphism in the regulation of bone metabolism in older men. A failure to find an association between the *CYP19* $(TTTA)_n$-repeat polymorphism and biochemical indices of bone turnover may be due to a lower precision of the latter markers. The lack of association with bone changes at the hip might be due to a lower precision of the latter BMD assessment at the hip compared with the forearm and/or possibly to the fact that the hip is a weight- bearing site. Furthermore, it should be emphasized that the results in the Merelbeke Study apply to a well-defined group of community-dwelling older men over age 70 year and that the strength of the study is that besides a complete data set of bone density, sex steroid levels and biochemical indices of bone turnover, it describes the detailed yearly longitudinal follow-up of BMD over a period of 4 year.

2.3.2 Effect of sex steroids on bone morphology at the hip

Areal bone mineral density (aBMD in gr/cm^2) as assessed by the 2-D projectional DXA is a parameter that integrates a variety of determinants, such as bone geometry and bone materials properties. Bone geometry has been shown to be a BMD-independent predictor of fracture. Derived from DXA acquisition data, estimations of structural parameters can be calculated based on mass profile curves. This methodology

resulted in the Hip Structural Analysis (HSA) program, that was developped by Tom Beck, a co-author in the manuscript reporting on the data in the Merelbeke Study. The relations between such derived geometry characteristics and sex-steroids were explored.
Within the limitations inherent to the applied methodology, the results illustrated that E_2 is associated not only with aBMD, but also with morphological variables at the narrow femoral neck as assessed by HSA. Both in the cross-sectional baseline analysis as well as in the longitudinal assessment, the data show a favourable effect of E_2 on the bone strength surrogates at the femoral neck. This observation argues for the previously reported association of higher E_2 levels with lower fracture risk in older men.
Although, there is only a limited decrease of serum estrogens with aging in men, we found that higher serum E_2 levels, within the observed physiological variation in older men, were prospectively associated with a significantly more favourable evolution of aBMD, average cortical thickness (avCT), section modulus (SM) and buckling ratio (BR). It can be hypothesized that a favourable evolution of structural parameters contributes, as a BMD-independent determinant, to the previously reported protective effect of E_2 levels on fracture incidence in men.

In the Merelbeke Study, significant correlations of T or bioT with aBMD and morphometric bone parameters are essentially restricted to the univariate cross-sectional analysis. This is in line with earlier data indicating that E_2 is more consistently related to maintenance of aBMD than T in older men and the present data suggest that this is also the case regarding the evolution of HSA assessed morphometric bone parameters in older men.

In the cross-sectional multivariate analysis, better physical fitness and higher E2 levels were associated with more favourable femoral neck bone strength surrogates; although the interaction term between these 2 determinants was not significant.
Increased bone turnover, as assessed by sCTX, weakly, but significantly contributed to higher endocortical diameter (ED) and BR values. This fits with the expectations that increased bone remodeling in the elderly leads to endocortical bone resorption and trabecularisation. However, no correlations of any bone turnover marker with longitudinal assessments of HSA parameters were observed. Intra- and inter-individual variations of the variables are quite substantial and probably leading to lack of power to document small effects

The HSA data could not detect the previously described (42) age-related increase in periosteal diameter (PD) in the cross-sectional analysis. However, in the longitudinal follow-up over 4 years the mean PD increase was 0.91% (SD: 2.69%). No significant relationships of PD with any determinant could be found. More specifically, T, E_2 nor their bioavailable fractions correlated with PD in the cross-sectional or longitudinal analysis.
The structural adaptation to age-related bone loss, e.g. the increase of periosteal perimeter might partially preserve the bending strength under physiologic loads, but cortical thinning (on average by 0.96% over the 4 years follow-up in the study) affects the buckling ratio (+ 2.47% increase / 4year), possibly increasing susceptibility to local buckling failure at the femoral neck in a fall.
IGF-1 levels have been reported to be associated with BMD and found to be involved in the sexual dimorphism in bone geometry occurring during aging. However, in the present study neither IGF-1 nor IGF-BP3 were associated with aBMD or HSA derived structural parameters.

The different relationships in the cross-sectional versus the longitudinal evaluation, may suggest the relative contributions of determinants to either bone formation and maturation versus age-related bone decline.
The HSA method is not a three-dimensional measurement but is based on two-dimensional DXA technology and relies on several assumptions about shape and trabecular distribution. Differences in rotational positioning between scans reduce precision, which are usually larger for geometric parameters compared to aBMD. To reduce reproducibility errors proximal femur DXA acquisitions were performed in duplo.
Femoral neck geometry parameters computed by HSA from DXA data correspond to parameters measured by QCT. But clearly, proximal femur breaking strength, estimated by engineering simulations on 2D DXA data is not as well correlated compared to the strength derived by a 3D finite element analysis (FEA) using QCT data. As bones are complex three-dimensional structures, there are inherent limits on the structural information which can be derived from two-dimensional areal projection DXA image data. So, interpretation of the presented relationships of E_2 with the geometrical femoral neck estimates, as contributors to the femoral neck fracture risk, must be done with caution.
The power of the Merelbeke Study may be limited for the detection of small changes, weak determinants or interactions between the determinants. There was no fracture endpoint in the longitudinal study. To aim for this, the study sample was too small and the follow-up time too short.

2.3.3 Cellular aging as reflected by telomere shortening: relation with skeletal aging in men.

In ambulatory living older men of the Merelbeke Study, telomere length was, although unrelated to baseline BMD, associated with prospectively assessed BMD changes at the distal forearm. Within the study population shorter average telomere lengths were associated with lower values of bone turnover. Yet, the association between short telomeres and higher bone loss appeared to be independent from bone turnover, since correction for bone turnover markers only increased the association.
As average telomere length appeared to be a possible candidate predictor for subjects at risk for bone loss and thus plausibly at risk for fractures at the old age, the Merelbeke findings are in line with the hypothetical role of mean peripheral blood cell telomere length as a biomarker of aging processes, also affecting bone homeostasis.
In agreement with the cross-sectional baseline data, no significant association between average telomere length and BMD were found within a small study on telomere length in postmenopausal women *(Kveiborg M et al, 1999)*.

In another cross-sectional study (Valdes AM et al, 2007) on a large number (n= 2150) of female twins from UK (mean age of 48 yr; range 18-80 yr) leucocyte telomere length was significantly correlated with BMD at the spine ($p<0.005$) and forearm ($p<0.013$), and showed a trend for association with BMD at the femoral neck ($p<0.06$) after adjustments for age, BMI, menopausal status, smoking and hormonal replacement therapy status. Longer telomeres were associated with reduced risk of osteoporosis (T-score < -2.5) at 2 or more sites (odds ratio = 0.594 (95% CI : 0.42-0.84; $p< 0.003$). In women over 50 years, osteoporosis was associated with 117 base pair shorter telomere length (TL) ($p<0.02$), equivalent to 5.2 years of telomeric aging. These data partly concur with our data from the Merelbeke study in which 84 elderly healthy men showed that shorter TL correlated with longitudinal forearm bone loss as assessed by DXA. The absence of a correlation in the cross-sectional Merelbeke data with the baseline BMD at the forearm and proximal femur (BMD at the spine was not assessed in the Merelbeke Study), might have been related to the small sample size or masking of this effect by more predominant long-term determinants of bone status in elderly men.
Further research is needed to test the causal pathways. A possible explanation for the association of telomere shortening and decrease BMD (if confirmed in additional large scale cohorts) is that leucocyte TL might register, at least in part, the cumulative burden of inflammation and oxidative stress during the individual' s lifetime (Saretzki G, Von Zglinicki T, 2002). Both processes have been implicated in bone loss (Genaldi L et al, 2005).

The finding of a significant relationship indicating increasing BMD loss at the forearm with smaller mean telomere restriction fragment (TRF) length has not previously been reported. Subjects with age-associated bone loss, as measured at the mid-region of the forearm, showed on average a 423 base pairs shorter mean TRF length as compared to subjects without bone loss. The lack of association between mean TRF length and BMD changes at the proximal femur may be due to the fact that the hip is a weight-bearing site, being influenced by muscle activity and gravity. Moreover, a larger variation coefficient of BMD data at the hip as compared to the forearm, by e.g. positioning problems in older male, may account for a lack of power to demonstrate a relationship in the smaller telomere length substudy.

A large prospective, age-stratified (65-70 yr, 70-75 yr, 75+ yr) study from Hong Kong examined the association between TL and BMD cross-sectionnally and the rate of bone loss over a 4-year period, in 1867 chinese elderly community living men and women. After adjustments for confounding factors, no association was observed with BMD or bone loss at the proximal femur. The decline in BMD was not reflected by corresponding changes in TL. In this study BMD at the spine and forearm was not measured. The cross-sectional and longitudinal data of this large Chinese study concur with our negative data for hip BMD from the small-sized Merelbeke study. The site dependent finding of inverse association between TL and bone loss at the forearm and not at the hip may result from factors other than biological aging. For example, mechanical factors may exert a greater influence on hip BMD compared with the forearm. Although the finding of the forearm bone loss following the age-associated telomere attrition in the Merelbeke study is unique, it fits in the theoretical concept of biological aging by reduced reserve capacity of cellular renewal.

Several studies of cultured osteoblasts have found intriguing links between observed telomere shortening and proliferation *in vitro*. Reconstitution of both osteoblasts and their precursors with ectopic telomerase activity extended their lifespan without loss of osteoblast formation potential in the latter *(Kveiborg M et al, 1999; Yudoh K et al, 2000; Yudoh K et al, 2001)*. Moreover, an experiment involving cultures of bone marrow stromal cells from young and older donors demonstrated that ageing is associated with a decreased proliferative capacity of osteoprogenitor cells, suggesting that a decreased osteoblastic cell number, and not a decreased osteoblastic function plays a central role in bone metabolism at an older age *(Stenderup K et al, 2003)*.

Despite the fact that a causal link between telomere attrition during fetal/early development and the occurrence of degenerative processes and chronic diseases later in life might be postulated, and given its impact on cellular ageing *(Jennings BJ et al, 1999, Bekaert S et al, 2004)*, we did not find an association between mean TRF length and baseline BMD in the Merelbeke Study. This might be explained by the replicative senescence paradigm *(Harley CB et al, 1990, Allsopp RC et al, 1992, Vaziri H et al, 1994 Blackburn EH, 2000)*. A decrease in proliferative capacity is the result of a stochastical mechanism by which the number of cells with critically short telomeres have increased gradually. This implicates that BMD at baseline is to a greater extent the consequence of influencing factors during a whole lifespan, while replicative senescence will be more apparent later in life and is therefore expected to be much more reflected through telomere length in BMD changes in the elderly.

Telomeres were found to shorten within peripheral blood cells with an average shortening rate of 23 bp per year of age in the older Merelbeke men. This is within the range of the previously reported average human adult telomere erosion rate estimated at 14-50 bp/year *(Slagboom PE et al, 1994, Vaziri H et al, 1994, Cawthon RM et al, 2003)*.
Several previous reports describe an association between steroid hormones and telomerase activity. Estrogens were shown to induce telomerase, e.g. in endometrial cells during the menstrual cycle *(Kyo S et al, 1999, Tanaka M et al, 1998)*, androgens on the other hand were reported to elicit opposite effects *(Soda H et al, 2000)*. We found no significant link between sex steroid status and telomere length in the older male population in Merebeke.
In conclusion, the Merelbeke Study revealed an intriguing association between telomere shortening and age-associated bone loss in ambulatory living older men. The results are indicative for possible involvement of cellular ageing in the mechanisms related to senile bone loss and suggest that telomere attrition rates might be a biomarker of this aging phenotype. Further prospective studies in subjects with a broader age range are needed to confirm the hypothetical role of telomere length as a biomarker of *in vivo* aging and of bone loss in particular.

2.4 Strengths and limitations of the Merelbeke study.

Strengths of the Merelbeke study:

The study population of the Merelbeke Study consists of a substantial cohort of ambulatory healthy aged men within a narrow age range of 70 to 85 years. Data on similar study populations specifically of generally healthy elderly men are scarce in the available literature. The number of subjects included, i.e. 352 without and 283 after exclusions, is close to the estimated target from a power calculation based on a pilot study in Merelbeke. Based on the calculated coefficient of variation of a dual-energy absorptiometry measurement, the number of subjects needed to demonstrate a yearly 1% decrease in BMD with a power of 80% was estimated at 300.
Besides the loss of follow-up because of death, the dropout in the Merelbeke was maintained within acceptable limits with the help of (1) dedicated logistic organisation of the investigations in a school located centrally in the community of Merelbeke, (2) the support of the municipal council and (3) the location of Merelbeke in close vicinity of the study coordinating Ghent University Hospital.
A strength of the Merelbeke study is the rather extensive clinical and biochemical evaluation of the study population and intensive yearly follow-up. Many of the published observations from the Merelbeke study, in a present total of 21 published papers on this study cohort, are corroborated by observations in other study cohorts, hereby supporting the representativeness of the Merelbeke study population.

Limitations of the Merelbeke study:

The relations found in the Merelbeke study between sex steroids levels and bone metabolism parameters when demonstrated, are generally weak. Several factors that may have contributed to limit the ability to demonstrate associations between studied variables include variability due to technical factors (e.g. suboptimal coefficients of variation for measurement of low hormonal concentrations) and factors contributing to biological variability (e.g. increasing prevalence with age of artefacts that may interfere with DXA measurements). Importantly, the size of the study population, although substantial, is still modest in comparison to more recently set-up very large study cohorts, such as the MrOs and the EMAS study cohorts, with numbers better suited to assess more subtle associations. Indeed, a particularity of the Merelbeke study is the inclusion of generally healthy men thus excluding markedly abnormal values. Nevertheless, as stated in the previous paragraph, many of the observations in the Merelbeke study population are supported by similar findings in other studies, providing an argument for the validity of this population study.
The size of the study population and the observed low number of incident fractures in the Merelbeke study precluded any analysis of incident fracture rates in relation with other parameters.
The mean values for the BMD loss in the Merelbeke study at the different measured sites are low and range between 0.04 to 0.44% / year. Rather low yearly rates of bone loss are not really unexpected in such an eldely population. Moreover, mean BMD changes fail to illustrate the reality of some men losing more bone whereas in others actually an increase of BMD is measured. It can be noted that the 0.39% yearly bone loss at the total hip observed by us in the Merelbeke study is very similar to that reported by Cauley et al (2010) for the MrOS study involving close to six thousand men and which, moreover, confirmed our findings of a negative association of bone loss with the serum levels of BioE2. The recruitment of (only) 47% from the total old male Merelbeke population could indeed induce a bias, even though compared to other population studies the 47% is an acceptable recruitment rate. "Selection of the fittest" is a well-known inevitable problem in studies on elderly populations. Moreover, this type of bias is further accentuated during longitudinal follow-up of elderly men. Therefore, it should be stressed that our findings apply primarily to healthy older men and that extrapolations to the general population of elderly men, with high prevalence of comorbidity, should be done with caution.

2.5 Future perspectives and research questions

The global aim of the Merelbeke Study was to find arguments pro or contra a role of age-related hormonal changes in men in clinical aging, in particular in "senile changes of bone homeostatis". There is no generally accepted definition of the so-called late-onset hypogonadism (LOH). This should include of course a specific T cutoff level plus co-existence of some of the clinical symptoms or manifestations of hypogonadism. These latter are of course not only subjective, but also difficult to discriminate from the symptoms of aging itself.

Although hypogonadism is a risk factor for male osteoporosis, the definition, and prevalence of hypogonadism in the community, causes (primary gonadal, hypothalamic, types of secondary hypogonadism), and structural consequence of hypogonadism are inadequately defined.
Although the finding from this thesis, confirmed or preceeded other studies on the primary associations of E_2 with bone metabolism, density and structure, many questions on the relationships of sex steroids with bone are still seeking for additional answers. Further clarification of the relative roles of T versus E_2 in bone homeostasis are needed. At what level of serum T or E2 is the bone integrity maintained and which interaction with e.g the somatotropic axis is operational?
What structural determinants of axial and appendicular bone strength are regulated by androgen, estrogen, growth hormone (GH), insulin-like growth factor 1 (IGF-1) (or their interactions)?
Is reduced bone size in men with spine or hip fractures due to failed growth- or age-related periosteal expansion? If reduced volumetric bone mineral density (vBMD) is due to reduced accrual, is this due to reduced cortical thickness? What factors regulate periosteal and endocortical modeling and remodeling? Are reduced trabecular numbers due to failed formation at the growth plate, excess resorption of primary trabeculae or reduced formation or excess resorption of secondary trabeculae? Is reduced trabecular thickness due to failed prepubertal or pubertal bone formation? What is the role of the relatively intense modeling/remodeling activity seen in young adult men?

Is reduced cortical and trabecular thickness during aging due to excessive endosteal resorption or reduced bone formation? If the former, is this due to increased remodeling sites or increased resorption depth?
Most evidence in men favours reduced formation and trabecular thinning as the cause of trabecular bone loss loss rather than increased resorption and loss of connectivity, as is the case in postmenopausal bone loss. Reduced bone formation may be due to reduced osteoprogenitors, reduced osteoblast matrix synthesis or early osteoblast apoptosis. Cortical bone loss is less than in women because endocortical resorption is less and periosteal apposition is greater. However, the gaps in our knowledge remain large.

To provide answers to this variety of questions, cross-sectional pilot studies and subsequently well-planned powered longitudinal follow-up studies should be performed. Powerfull technologies should be introduced to study in more detail bone strength aspects as bone structure/morphology, e.g. by high resolution QCT and bone material quality assessments, e.g. by detailed histologic, nano-indentation techniques, …

It should be acknowledged that presently improved and more sensitive methods for the measurements of serum sex steroid levels are available (mass spectrometry). However, the added value of the new accepted standard needs to be proven in men, in whom sex steroids levels do not reach the extremely low levels as observed in postmenopausal women.

In the last decades, bone has been acknowledged as the organ obeying to the muscle. Muscle activity is the most important origin of the mechanical forces applied to the bone. Bone adapts to its usage and it should be remembered that this principle applies on a skeletal regional basis. The muscle-bone relationship is a logic consequence from the bone mechanostat concept, that was conceptualized and described by Harald Frost in the eighties *(Frost H, 1987)*. The molecular and cellular basis of this concept, including its control feedback loops have been partially uncovered.

The systemic modulations of this strongly locally regulated mechanism, by e.g. sex steroids, growth hormone axis,, … have been studied in the Merelbeke study. The effects have been generally spoken weak. To further explore the systemic modulation of the mechanostat set points, observational and interventional studies could add to understand the interaction of locally applied forces and locally measured bone parameters.

This insight will be important in the future, as frailty and sarcopenia are now recognized as major indicators of the aging process. The insight gathered from the bone fields brings now into the field determinants of muscle metabolism and its neurological and other control systems. In this respect the interplay between the bone strength evaluation and the fall risk assessment, is of the most clinical relevance to optimize fracture prediction.

Genetic discoveries have provided significant contributions to new insights into bone metabolism. And more has yet to come … . GWAS consistently indicated the important loci regions, however the complexity of the interrelation between potential candidate determinants is still to great, to expect imminent break-throughs, which could be of clinical importance. Also the so far identified genes explain only a fraction of the genetically determined between subject differences in bone phenotypes.

To be engaged in the topic in the next decades, the Unit of Osteoporosis and Metabolic Bone Diseases in the Ghent University Hospital has started to collect detailed phenotypic description of bone-related characterisctics of two male cohorts, from which already important results have been published and presented in some doctoral thesis contributions.

The importance of the genetic backgroud for osteoporosis, as well as for all other diseases, came to the foreground. The two extensive male study populations were recruited to investigate heritability of bone characteristics and the interaction with lifestyle-related and hormonal factors.

The first one was recruited from the consultation of osteoporosis and metabolic bone diseases. Male probands with idiopathic osteoporosis as determined by low bone density and/or vertebral fragility fractures, were invited to participate in a Family Male osteoporosis Study (FAMOS). Approximately thousand participants are recruited in this “three generation study”; including children down to 6 years of

age. This will provide the opportunity to investigate combined effect of a variety of parameters through the pubertal transition.
As second study cohort recruited in respect to planned genetic research, was a population-based study of young adult males at their peak bone mass age. Brother pairs were invited from several suburbs surronding the area of the Ghent University Hospital. Started as a cross-sectional study in the early years of this century, a second round 8 to 10 years after the first visit is planned in 2014 to 2016, with the introduction of some new techniques of bone evaluations and collection of additional genetic materials.

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Summary :

This thesis provides insights on the contributions of sex steroids to bone metabolism in older male. To explore this topic bone densitometry by dual X-ray absorptiometry (DXA) is the gold standard tool. It can measure areal bone mineral density (aBMD), which is one of the most usefull bone characteristics for evaluation in a clinical setting. BMD integrates a number of bone properties (i.e. bone mass, bone size and geometry, bone material and mineral quality) into a single number (g/cm^2). The reason why this parameter evolved to be the standard since many years is its most optimal approximation of bone strength and its predictive capacity for incident fragility fractures. Moreover, DXA is (or can be) widely available, uses very low doses of irradiation, and is easy to use in clinical practice. Later on, other bone evaluation techniques and parameters have been added to the panel of investigations, but they contributed, although significantly, only little to the strength of integrated BMD value.
Decreased bone health and consequent fragility fractures in men are a public health problem. About one third of all hip fractures occur in men. The prevalence of spine fractures is about half that of women.
Interestingly, spine and hip fracture incidence rises markedly with aging. The incidence in men equals that in women at approximately 7 to 10 years older age. Generally, the higher mortality and fewer falls in men may also contribute to the lower probability of fractures in men. The increasing longevity in men is likely to increase the public health burden of fractures in men.
The causes of the higher mortality in men than in women following hip or spine fracture are not well defined. Men are much less likely to be diagnosed and/or treated for osteoporosis. Until osteoporosis in men is increasingly recognized by both medical professionals and the general public, it is doubtful that the fracture rate will decrease in men.

The results presented in this thesis were derived from a cross-sectional study and longitudinal follow-up data collected from a cohort of community-dwelling older males in a well-defined community in the late nineties. In men, there is no dramatic decrease in sex steroid secretion, analogous to the radical changes during menopausal transition in women. Our study and others, in older men have demonstrated that serum estradiol levels, mostly derived from aromatization of testosterone, are inversely and more closely, related to osteoporosis indicators like bone density and fracture than testosterone levels.

The relation between bone health and sex steroids in men is complex. In animal models and in humans, there is evidence for direct effects on bone by both androgens and estrogens. In men estradiol is mainly derived via aromatization of testosterone in a variety of tissues. There are both genetic and environmental effects on aromatase activity.

The Merelbeke study contributed insights on whether the decreases in serum testosterone and free testosterone with aging have any impact on osteoporosis and fracture risk. Its cross-sectional and longitudinal analyses did not support this relation. Besides the idiopathic form, many reasons for secondary osteoporosis in men can be documented by clinical and laboratory evaluation. While treatment of an underlying secondary cause of osteoporosis may improve bone mineral density and fracture risk, additional and specific osteoporosis treatment may be required. Methods for evaluation and treatment have been reviewed.

In the thesis contributions to the diagnostic evaluations of osteoporosis in men are provided **(Chapter 3.1 and Chapter 3.2.)**.

Standardization of the DXA BMD evaluation **(chapter 3.1)** is important, as differences in the applied techniques are quite substantial. The manufacturers of bone densitometry instruments do not use the same methods for the data acquisition, the bone edge detection and mass estimation algorythms are different. Consequently, the absolute BMD values generated are brand specific. Expression of the data in percentage or standard deviation score referring to a reference population, does not resolve the issue, as the reference populations applied in the different DXA brands are different.

We contributed to the standardization of DXA data expression in men, by applying the conversion formulae derived from female studies to a sample of young healthy Belgian males. This unique, nation-wide reference sample avoids the inconsistencies that result from the use of different, manufacturer-derived reference

populations. The comparison of the generated Belgian reference values with internationally accepted standard (i.e. NHANES III) confirmed the adequacy of the NHANES values for the Belgian sample.

The standardization efforts in the reported Belgian male study **(Chapter 3.1)** are important in respect to generate a uniform framework for the diagnosis and treatment of osteoporosis in men. By calculating standardized BMD values and by providing national reference values, osteoporosis can be diagnosed uniformally across different centers using different DXA devices.

DXA based BMD at the proximal femur is a standard reference for the diagnosis of osteoporosis and an important factor for the calculation of the absolute fracture risk in women and men. Other bone evaluations (by single beam bone densitometry or DXA or quantitative ultrasound (QUS)) at peripheral sites (forearm, tibia or heel) in the older men of the Merelbeke Study population **(Chapter 3.2)** performed only moderately in predicting standard DXA based central (i.e. at the hip) BMD. Possible reasons for discrepancies are: 1) site-specific processes during skeletal development and/or aging; 2) accuracy biases in data acquisition; 3) different bone geometry according to the bone site; 4) measurement technique and/or patient positioning.

Comparing the efficacy of the various more practical peripheral measurements (less expensive, mobile, low or no irradiation) in the Merelbeke study in predicting a "gold standard" such as the hip BMD revealed that all these approaches performed equally well.
The value of the peripheral bone assessments in clinical practice has to be proven by cost-effectiveness analyses. These methods can be an alternative approach in regions where central DXA is not available. Furthermore, it was shown in the Merelbeke study **(Chapter 3.2)** that peripheral bone evaluation techniques, are less reproducible and therefore were inadequate to be used as a follow-up parameter in the DXA BMD sized Merelbeke study.

In the Merelbeke Study the assessed bone turnover indices of older community-dwelling males **(Chapter 4)** showed lower values compared to young adult male controls, but higher values compared to a middle-age reference group. A rather limited and non-consistent increase of bone turnover markers was observed in the elderly. Prevailing bone turnover parameters were negatively associated with areal BMD in the older male study subjects, comparable to such relationships reported in postmenopausal women.

Concerning the sex steroid levels, approximately 50% lower free testosterone (FT) and 70% lower DHEAS were found as compared to the young reference group. The relatively well-preserved total E_2 levels probably reflect the increased aromatisation of the lower T reserve. The observed decrease free E_2 (FE_2) and bioavailable E_2 ($bioE_2$), a consequence of increased SHBG was significant, but limited (12%).

It was demonstrated that bone turnover parameters in the older men in the Merelbeke Study were marginally affected by differences in sex steroid levels. The associations in both univariate and multivariate analyses were weak and inconsistent, and even totally absent for T. These older male data do not support the view that the observed positive correlations between sex steroid levels and BMD are mediated by bone turnover.

It should be emphasized that the strength of the Merelbeke Study is that it applies to a well-defined group of community-dwelling older men over age 70 year and covers a complete data set of bone density, sex steroid levels and biochemical indices of bone turnover, in both a cross-sectional setting and a detailed yearly longitudinal follow-up of BMD over a period of 4 year.

In community-dwelling older men in Merelbeke, $bioE_2$ was consistently associated with BMD changes at all measured sites **(Chapter 5.1)**. Higher estradiol levels were followed with smaller BMD losses during the longitudinal follow-up. The *CYP19* $(TTTA)_n$-repeat polymorphism was, moreover, an additional independent determinant of BMD changes at the distal forearm. The Merelbeke Study, confirmed and extended previous finding in other studies on the role of estrogens in the maintenance of bone mass in men. In contrast to some of these, our data did not support the existence of a threshold phenomenon for the relation between serum $bioE_2$ and bone loss. The analyses rather illustrated a continuous relationships between sex steroids levels and bone turnover markers with BMD.

In the Merelbeke Study no relationship between the *CYP19* genotype and circulating levels of $bioE_2$, bioT or LH were found, nor with the T/E_2 ratio **(Chapter 5.1)**. Whereas there was no correlation between the *CYP19*

genotype and prevalent BMD, the *CYP19* genotype was associated with %BMD change at the distal forearm in both univariate and multivariate analyses. The data suggest a possible local effect of the *CYP19* genotype at the bone tissue level. Tissue-specific regulation of aromatase enzyme expression has been reported also for other tissues. The failure to find an association between the *CYP19* $(TTTA)_n$-repeat polymorphism and biochemical indices of bone turnover could be due to a lower precision of the latter markers or the limited sample size of Merelbeke Study **(Chapter 5.1)**.

Only 8.4% of the Merelbeke study subjects reported having sustained a fracture after the age of 50 and only a few fracture have been registered during the 4 year follow up. This precluded any meaningfull analysis of the relations with the sex steroids.

Bone geometry has been shown to be a BMD-independent predictor of fracture. Derived from DXA acquisition data, estimations of structural parameters can be calculated based on mass profile curves. This methodology resulted in the Hip Structural Analysis (HSA) program. It was developed by Tom Beck, a co-author in the manuscript reporting on the HSA data in the Merelbeke Study **(Chapter 5.2)**.
Within the limitations inherent to the applied methodology, the results illustrated that E_2 is associated not only with aBMD, but also with morphological variables at the narrow femoral neck as assessed by HSA. Both in the univariate and multivariate cross-sectional baseline analysis as well as in the longitudinal assessment, the data show that higher E_2 is associated with more favourable bone strength surrogates at the femoral neck.

In the Merelbeke Study, significant correlations of T or BioT with aBMD and morphometric bone parameters are essentially restricted to the univariate cross-sectional analysis. Some differences in the observed relationships in the cross-sectional versus the longitudinal evaluations may suggest the relative contributions of determinants to either bone formation / maturation versus age-related bone decline.

In contrast to what was previously shown by our research group on pQCT data in a young adult male population, an interaction of between E_2 and physical activity was not significant for the bone morphology parameters at the femoral neck in the older Merelbeke Study population.

Increased bone turnover, as assessed by sCTX, was weakly related to higher endocortical diameter (ED) and buckling ratio (BR) values. This relationship was not found in longitudinal assessments of HSA parameters. Intra- and inter-individual variations of the variables were quite substantial and probably leading to lack of power to document small effects.

Next to the age-related bone loss, age-related increase of periosteal perimeter is explained as a structural adaptation to partially preserve the bending strength under physiologic loads. In the longitudinal follow-up over 4 years an increase of 0.91% (SD: 2.69%) of the mean periosteal diameter (PD) was documented. No significant relationships of PD with any determinant could be found in the cross-sectional or longitudinal analyses.

The bone loss in terms of cortical thinning (on average by 0.96% over the 4 years follow-up in the Merelbeke Study) affects the buckling ratio (+ 2.47% increase / 4year), possibly increasing susceptibility to local buckling failure at the femoral neck in a fall.
In contrast to the reported association of IGF-1 levels with BMD and sexual dimorphism in bone geometry occurring during aging, neither IGF-1 nor IGF-BP3 were associated with aBMD or HSA derived structural parameters in the older Merelbeke Study population **(Chapter 5.2)**.

As bones are complex three-dimensional structures, there are inherent limitations on the structural information, which can be derived from two-dimensional areal projection DXA image data. So, interpretation of the presented relationships of E_2 with the geometrical femoral neck estimates as documented in the Merelbeke Study, must be done with caution.

In ambulatory living older men of the Merelbeke Study, telomere restriction fragment (TRF) length **(Chapter 5.3)** was associated with prospectively assessed BMD changes at the distal forearm. Telomeres were found to shorten within peripheral blood cells with an average shortening rate of 23 bp per year of age in the older Merelbeke men. The subjects with age-associated bone loss, as measured at the mid-region of the forearm, showed on average a 423 base pairs shorter mean telomere restriction fragment (TRF) length as compared to subjects without bone loss. Shorter telomere lengths were also associated with lower values of bone turnover.

However, the association between short telomeres and higher bone loss appeared to be independent from bone turnover.

We did not find an association between mean TRF length and baseline BMD in the Merelbeke Study **(Chapter 5.3)**. Most probably, BMD at baseline is to a greater extent the consequence of influencing factors during a whole lifespan. The replicative senescence of the bone cells will be more apparent later in life and as reflected, through telomere length, results only in BMD decrease at the older age.
As opposite to androgens, estrogens induce telomerase activity. However, we did not find an association between sex steroid status and telomere length in the older male population in Merebeke.
These results for telomere length are indicative for possible involvement of cellular ageing in the mechanisms related to senile bone loss. Further prospective studies in subjects with a broader age range are needed to confirm the hypothetical role of telomere length as a biomarker of *in vivo* aging and of bone loss in particular.

The global aim of the Merelbeke Study was to find arguments pro or contra a role of age-related hormonal changes in men in clinical aging, in particular in "senile changes of bone homeostasis". There is no generally accepted definition of the so-called late-onset hypogonadism (LOH). This should include of course a specific testosterone cutoff level plus co-existence of some of the clinical symptoms or manifestations of hypogonadism. These latter are of course not only subjective, but also difficult to discriminate from the symptoms of aging itself. Although the findings in this thesis, confirmed or preceeded other studies demonstrating that in men among sex steroids estradiol is most consistently associated with bone metabolism, -density and -structure, many questions on the relationships of sex steroids and bone are still seeking for additional answers.

To provide answers to many outstanding questions on bone homeostasis and health in men, well-planned and powered longitudinal follow-up studies are needed. More powerfull technologies, compared to only the DXA, that was available at the time of the Merelbeke Study, should be introduced to study, in more detail, bone strength aspects such as bone structure/morphology, e.g. by high resolution QCT and bone material quality assessments, e.g. by detailed histologic, nano-indentation techniques, …

In recent years large international cohort studies on bone health in men have been initiated (e.g. the MrOS study). At the "Unit of Osteoporosis and Metabolic Bone Disease" in the Ghent University Hospital two large population studies have been set up to answer some of these question.
The first study concerns the FAMOS Study (FAMily Osteoporosis Study), including 3-generaton family pedigrees starting from male probands with idiopathic osteoporosis.
Secondly, the SIBLOS Study (SIBLing Osteoporosis Study) consisting of a population-based recruitment of brother pairs at the age of peak bone mass.

Samenvatting :

Deze thesis verstrekt inzichten over de effecten van de sex hormonen op het botmetabolisme bij oudere mannen. Botdensitometrie door dubbele energy X-stralen absorptiometrie (DXA) is een gouden standaard om dit onderwerp te onderzoeken. Met deze technologie wordt de botmineraal densiteit (BMD) bepaald. BMD is één van de meest bruikbare parameters voor de evaluatie van het bot vanuit een klinische vraagstelling. BMD weerspiegelt verschillende eigenschappen van het bot : de botmassa, de botgrootte en botgeometrie, de botmateriaal en -mineraal kwaliteit. BMD wordt uitgedrukt door één cijfer (g/cm^2). De reden waarom deze parameter beschouwd wordt als een "standaard" is gebaseerd op het feit dat het de meest optimale benadering is voor evaluatie van botsterkte en dat deze predictief is voor fragiliteitsfracturen. Daarenboven is DXA ruim toegankelijk, gebruikt het lage stralingsdosissen en is eenvoudig toepasbaar in de klinische praktijk. Later werden andere botevaluaties aan het panel van onderzoeken toegevoegd. Hun bijdrage tot de inschatting van de botsterkte, is significant, doch beperkt ten opzichte van één geïntegreerd DXA BMD cijfer.

Een slechte botgezondheid en begeleidende botfracturen bij mannen vormen een belangrijk problem voor de volksgezondheid. Eén derde van alle heup fracturen gebeurt bij mannen. De prevalentie van wervelfracturen bij mannen is de helft van deze bij vrouwen. De incidentie van wervel- en heupfracturen stijgt aanzienlijk met de leeftijd. De incidentie bij mannen bereikt deze bij vrouwen op een 7 à 10 jaar oudere leeftijd. De hogere algemene mortaliteit en het minder frekwent vallen bij mannen, zijn 2 factoren die tot de lagere fractuurkans bij mannen leiden. De trend tot stijging van de levensverwachting bij de man zal een toename betekenen voor gezondheidskosten door botfracturen. De oorzaken van een hogere mortaliteit na wervel- of heupfractuur bij de mannen ten opzichte van de vrouwen zijn niet goed gekend. Bij een man is de kans dat de diagnose van osteoporose wordt gesteld en/of dat hiervoor therapie wordt opgestart laag. Zolang osteoporose bij mannen niet in toenemende mate wordt herkend door de gezonheidswerkers en het publiek, is het onwaarschijnlijk dat de fractuurincidentie bij mannen zal dalen.

De resultaten die voorgesteld worden in deze thesis zijn bekomen door een cross-sectionele studie en longitudinale opvolging van een cohorte van oudere mannen. Deze mannen waren ambulant en woonden zelfstandig bij de start van de studie in de gemeente Merelbeke. Het onderzoek werd uitgevoerd eind de jaren negentig.

Bij de veroudering bij mannen is er geen dramatische daling van de bloedspiegels voor sexhormonen, analoog aan de radicale veranderingen tijdens de menopausale transitie bij vrouwen. De Merelbeke Studie en andere studies hebben aangetoond dat serum oestradiol, voornamelijk afkomstig door aromatisatie van testosteron, sterker negatief geassocieerd is met indicatoren van osteoporose (zoals botmineraaldensiteit en fractuur) dan testosteron.

De relatie tussen botgezondheid en sex steroiden bij mannen is complex. In diermodellen en bij mensen is er evidentie voor directe effecten van androgenen èn oestrogenen op het bot. Bij mannen is oestradiol voornamelijk afkomstig van de aromatisatie van testosteron in een aantal weefsels. Zowel genetische als omgevingsfactoren beïnvloeden de aromatisatie.

De Merelbeke studie heeft bijgedragen bij tot inzichten over de invloed van de leeftijdsgebonden daling van serum totaal testosteron (T) en vrij testosteron (FT) op het ontstaan van osteoporose. De cross-sectionele en longitudinale analyses uit de Merelbeke Studie ondersteunen een dergelijke relatie niet. Naast de idiopathische vorm, zijn er vele oorzaken van secundaire osteoporose bij de man. Deze kunnen gedocumenteerd worden door klinische en biochemische evaluaties. Uiteraard zal de behandeling van de onderliggende aandoening(en) de botmineraaldensiteit verbeteren en het fractuurrisico doen dalen. Additioneel is hier ook een specifieke osteoporose behandeling nodig. De diagnostische methoden en behandelingen voor osteoporose bij de man werden uitgebreid besproken.

In de thesis wordt een bijdrage geleverd tot de diagnostische evaluatie van osteoporose bij mannen **(Chapter 3.1 and Chapter 3.2.)**.

Standardisatie van DXA BMD evaluatie **(chapter 3.1)** is belangrijk, omdat er substantiële verschillen bestaan tussen de gebruikte technieken. De producenten van toestellen voor botdensitometrie gebruiken

verschillende methoden voor het verwerven van de meetgegevens, de aflijning van het geprojecteerde botoppervlak en het algoritme om de weefselmassa te bepalen. Bijgevolg zijn de bekomen absolute BMD waarden producent specifiek. De uitdrukking van BMD in percentages of standarddeviatie afwijkingen ten opzichte van een referentie populatie lost dit probleem niet op, omdat de gebruikte referentie populaties ook producent specifiek zijn.
Om tot een standardisatie of DXA BMD gegevens bij mannen te komen hebben we de conversie formules, die voor vrouwen werden opgesteld, toegepast om een populatie jonge gezonde Belgische mannen. Deze unieke Belgische referentie populatie vermijdt verschillen die resulteren van het gebruik van producent specifieke referenties. De vergelijking van deze Belgische referentie data met internationaal aanvaarde standaarden (b.v. NHANES III) bevestigt de adekwaatheid van de NHANES waarden voor de Belgische populatie.

De voorstellen van standardisatie die aangereikt worden in de studie van Belgische mannen **(Chapter 3.1)** zijn belangrijk om een uniforme diagnose en therapie voor osteoporose bij mannen toe te laten. Door het gebruik van een gestandardiseerde BMD waarde en verwijzing naar uniforme nationale referentiewaarden, kan osteoporose op een uniforme wijze gediagnosticeerd worden, zelfs bij gebruik van verschillende DXA apparaten.

DXA BMD ter hoogte van de proximale femur is de referentie voor de diagnose van osteoporose en is een belangrijke factor in de berekening van het absoluut fractuurrisico bij man en vrouw. Andere botevaluatie-technieken ter hoogte van perifere botregio's werden gebruikt in de evaluatie van de oude mannen uit de Merelbeke Studie **(Chapter 3.2)**. De predictieve waarde van perifere X-stralen absorptiometrie of kwantitatieve ultrasonografie ter hoogte van voorarm, tibia of hiel voor de standaard centrale DXA ter hoogte van de heup was matig. Mogelijke verklaringen voor de discrepante bevindingen zijn : 1) plaats-specifieke effecten tijdens ontwikkelings- en/of verouderingsprocessen van het skelet, 2) verschillen in de accuraatheid van de dataverwerving, 3) verschillen in de botgeometrie volgens de localisatie of 4) verschillen in de meettechnieken en/of positionering van de proefpersoon.

Onderlinge vergelijking in de Merelbeke Studie van de efficiëntie van de verschillende perifere botmetingen, die een lagere kostprijs hebben, mobiel zijn en een lagere of geen straling gebruiken, ter predictie van de goud standaard, zoals de heup BMD, toonde dat alle perifere metingen evenwaardig waren. De waarde van de perifere botmetingen in de klinische praktijk moet aangetoond worden via een kost-effectiviteitsanalyse. Deze methoden kunnen een alternatieve benadering vormen wanneer centrale DXA niet beschikbaar is. Er werd in de Merelbeke Studie aangetoond **(Chapter 3.2)** dat de perifere botmetingen minder reproduceerbaar waren. Daarom werden deze niet gebruikt als follow-up parameters in de Merelbeke Studie.

In de Merelbeke Studie toonden de biochemische parameters van botombouw voor de studiepopulatie oudere mannen lagere waarden dan de jonge gezonde controle personen **(Chapter 4)**, maar hogere warden in vergelijking met mannen van middelbare leeftijd. Een beperkte en niet consistente stijging van de botturnover parameters werd vastgesteld met toenemende leeftijd in de oude mannelijke populatie. Zoals gerapporteerd bij postmenopausale vrouwen, waren de botombouw parameters negatief gecorreleerd met BMD.

Bij de oudere mannen in Merelbeke was vrij testosteron 50% lager en DHEAS 70% lager in vergelijking met de jonge referentie groep. De relatief goed behouden serumconcentraties van E_2 weerspiegelen waarschijnlijk een toegenonmen aromatisatie van de lagere testosteron reserve. De daling in het vrij oestradiol (FE_2) en biologisch beschikbare oestradiol ($bioE_2$) die het gevolg zijn van een stijging van de SHBG concentratie was significant, doch beperkt (12%).

De botombouw parameters in de oudere mannen uit de Merelbeke Studie waren minimal beïnvloed door verschillen in spiegels van sex hormonen. De associaties in de univariate en multivariate analyses waren zwak en inconsistent en zelf volledig afwezig voor testosteron. Deze data vormen geen ondersteuning voor de visie dat de botombouw een mediërende rol zou spelen in de vastgestelde positieve correlaties tussen de sexhormonen en de BMD.

De sterkte van de Merelbeke Studie is dat het een goed omschreven populatie van ambulant-wonende mannen ouder dan 70 jaar betreft en een complete data set omvat van botdensitometrie, sex hormonen, en botombouw parameters. De data werden geanalyseerd in een cross-sectionele evaluatie bij de start en

na een gedetailleerde jaarlijkse follow-up over een periode van 4 jaar.

Bij de ambulante oudere mannen in Merelbeke, was bioE_2 consistent geassocieerd met BMD veranderingen ter hoogte van de verschillende meetplaatsen **(Chapter 5.1)**. Hogere E_2 spiegels werden gevolgd door kleinere BMD verliezen tijdens de longitudinale opvolgingsperiode. Het *CYP19* $(TTTA)_n$-repeat polymorfisme was een additionele onafhankelijke determinant van de BMD veranderingen ter hoogte van de distale voorarm. De Merelbeke Studie bevestigde de bevindingen van andere studies over de rol van oestrogenen in het behoud van de botmassa bij de verouderende man en breidde ze verder uit. In tegenstelling tot sommige van de eerdere bevindingen, kunnen de gegevens uit de Merelbeke Studie echter niet bevestigen dat er een drempelfenomeen zou bestaan in de relatie tussen bioE_2 en botverlies. De verrichte analyses suggereren eerder het bestaan van continue relaties tussen de sexhormonen, botombouw parameters en BMD.

Er werden geen relaties gevonden tussen *CYP19* genotypes en de circulerende spiegels van bioE_2, bioT of LH, noch met de T/E_2 ratio **(Chapter 5.1)**. Terwijl er geen correlatie was tussen *CYP19* genotypes en de prevalente BMD, was het *CYP19* genotype, bij zowel de univariate als de multivariate analyses, geassocieerd met het botverlies ter hoogte van de voorarm. Deze gegevens suggeren een mogelijk effect van het *CYP19* genotype op het niveau van het botweefel zelf. Een weefsel-specifieke controle van de expressie van het aromatase enzyme werd ook al gerapporteerd in andere weefsels. Het ontbreken van een associatie tussen het *CYP19* $(TTTA)_n$-repeat polymorfisme en de biochemische indicatoren van botombouw zou kunnen verklaard worden door de lagere reproduceerbaarheid van de botombouw parameters of door de beperkte omvang van de studiepopulatie in de Merelbeke Studie **(Chapter 5.1)**.

Slechts 8.4% van de Merelbeke Studie deelnemers gaf aan ooit een botbreuk gehad te hebben na de leeftijd van 50 jaar. Tijdens de 4 jaar opvolging werden slechts enkele nieuwe botfracturen gerapporteerd. Hierdoor was het niet zinvol om analyses in relatie tot de sexhormonen uit te voeren.

Het is aangetoond dat botgeometrie een BMD-onafhankelijke voorsteller is van het fractuurrisico. Vanuit DXA data-acquisitie gegevens kan op basis van de massaprofielen een berekening van structurele parameters gebeuren. Deze methodologie heeft geleid tot het "Hip Structural Analysis (HSA)" algoritme. Dit werd ontwikkeld door Tom Beck, die ook co-auteur is van het manuscript dat de resultaten van de HSA analyses van de Merelbeke Studie rapporteert **(Chapter 5.2)**.
Binnen de beperkingen, inherent aan de HSA evaluatie, tonen de resultaten dat E_2 niet alleen geassocieerd is met BMD, maar ook met morfologische variabelen ter hoogte van de femurhals. Zowel in de univariate en multivariate cross-sectionele als in de longitudinale analyses, is een hogere E_2 spiegel geassocieerd met gunstigere warden voor surrogaat parameters voor botsterkte ter hoogte van de femurhals.
In de Merelbeke Studie waren de significante correlaties van T of bioT met BMD en de morfologische botparameters beperkt tot de univariate cross-sectionele evaluatie. Sommige verschillen in de geobserveerde relaties in de cross-sectionele versus de longitudinale evaluaties verwijzen mogelijk naar contributie van determinanten van botformatie en -maturatie en anderzijds van factoren betrokken bij het leeftijdsgebonden botverlies.

In tegenstelling tot wat werd aangetoond door onze onderzoeksgroep met perifere kwantitatieve computer tomografie bij jonge mannelijke volwassenen, is de interactie tussen E_2 en fysieke activiteit bij de oudere mannen uit de Merelbeke Studie was niet significant voor de botmorfologische parameters ter hoogte van de femurhals.

Een verhoogde botombouw, zoals bepaald door serum crosslink telopeptiden (sCTX), was zwak gecorreleerd met grotere endocorticale diameter en buckling ratio. Deze relaties werden niet teruggevonden in de longitudinale evaluaties van de HSA parameters. De intra- en interindividuele variaties waren vrij substantieel en mogelijk leidend tot een gebrek aan statistische kracht om kleine effecten te kunnen aantonen.

De leeftijdsgebonden toename van de periosteale perimeter wordt beschouwd als een structureel adaptatiemechanisme voor de leeftijdsgebonden afname van de botmassa Zo wordt de botsterkte partieel behouden onder fysiologische belastingen. Bij de longitudinale follow-up in de Merelbeke Studie werd een 0.91% (SD: 2.69%) toename van de gemiddelde periosteale diameter (PD) gedocumenteerd. Er werden echter geen significante relaties van de PD met enige determinant vastgesteld in de cross-sectionele noch de longitudinale analyses.

De vermindering van de corticale dikte (ongeveer 0.96% over 4 years opvolging), als deel van het verlies aan botmassa, bepaalt grotendeels de toename van de buckling ratio (+ 2.47% / 4year). Hierdoor wordt de vatbaarheid voor lokale buckling ter hoogte van de femurhals tijdens een val verhoogd.
In tegenstelling tot gerapporteerde associaties van IGF-1 met BMD en het geslachtsgebonden dimorfisme van botgeometrie optredend bij de veroudering, was noch IGF-1 noch IGF-BP3 geassocieerd met BMD of de HSA structurele parameters in de Merelbeke Studie **(Chapter 5.2)**.

Daar beenderen complexe drie-dimensionele structuren zijn, leidt dit tot inherente beperkingen van de structurele informatie die kan bekomen worden uit twee-dimensionale projectie gegevens van DXA. De interpretatie van de relaties van E_2 met de geometrische inschattingen van de femurhals moet dus met enige voorzichtigheid worden gedaan.

Bij de ambulant-wonende oudere mannen uit de Merelbeke Studie, was de telomeer restrictiefragment (TRF) lengte in de perifere bloedcellen **(Chapter 5.3)** geassocieerd met het longitudinal vastgesteld botverlies ter hoogte van de voorarm. Telomere lengte verkortte in de Merelebeke Study met een tempo van 23 base-paren per leeftijdsjaar. De oudere mannen met een leeftijdsgebonden botverlies ter hoogte van de mid-voorarm toonden een telomeer lengte die gemiddelde 423 base-paren korter was dan de mannen die geen botverlies toonden. Een kortere telomeerlengte was ook geassocieerd met lagere waarden voor de parameters van botombouw. Nochtans, was de associatie tussen de kortere telomeren en het hogere botverlies onafhankelijk van de botombouw.

We vonden geen associatie tussen de gemiddelde TRF lengte en de BMD bij aanvang van de Merelbeke Studie **(Chapter 5.3)**, meest waarschijnlijk omdat BMD een resultante is van verschillende factoren gedurende de ganse levensloop. De verouderingsverschijnselen in de botcellen zal duidelijker worden op oudere leeftijd, zoals ondersteund wordt door de relatie van de telomeer lengte en botverlies enkel op oudere leeftijd.

In tegenstelling tot de androgenen, induceren oestrogenen telomerase activiteit. Nochtans vonden we geen associatie tussen de sex hormonen en de telomeerlengte in de oudere mannen in Merelbeke. Deze resultaten over de telomere lengte vormen aanwijzingen voor de betrokkenheid van cellulaire veroudering in het ontstaan van leeftijdsgebonden botverlies. Verdere prospectieve studies in populaties met verschillende leeftijdsgroepen zijn nodig ter bevestiging van de hypothetische rol van de telomeerlengte als bio-merker van veroudering en in het bijzonder van botverlies.

De algemene doelstelling van de Merelbeke Studie was het zoeken naar argumenten pro of contra de invloeden van leeftijdsgebonden hormonale veranderingen bij oudere mannen, in het bijzonder deze op de botehomeostase. Er is geen algemeen aanvaarde definitie van het zogenaamde “late-onset” hypogonadisme LOH). Deze definitie moet natuurlijk een specifieke testosteron drempelwaarde én het gelijktijdig bestaan van sommige klinische symptomen of tekenen van hypogonadisme omvatten. Deze symptomen of tekenen zijn natuurlijk niet alleen subjectief, doch ook moeilijk te onderscheiden van de symptomen van de veroudering zelf. Alhoewel de bevindingen in deze thesis de consistente relaties van oestradiol met botmetabolisme, botdensiteit en botstructuur uit andere studies bij mannen bevestigen of voorafgaan, blijven er vele vragen over de relatie van sex hormonen en bot nog onbeantwoord.

Om antwoorden te formuleren op deze vragen over bothomeostase en botgezondheid bij mannen zijn er bijkomende longitudinale onderzoeken met een goede opmaak en steekproefgrootte nodig. Nieuwere en krachterige technieken, die niet beschikbaar waren in de tijd van de Merelbeke Studie, moeten geïntroduceerd worden. Deze technieken, zoals hoge resolutie QCT, specifieke bothistologie en nano-indentatie, kunnen naast de DXA BMD, verschillende andere aspecten van botsterkte evalueren, namelijk de botstructuur, botmorfologie en botmateriaal kwaliteit.

In de voorbije jaren werden grootschallige internationale cohort studies over de botgezondheid bij mannen opgezet (bv. MrOS studie). In de “Eenheid voor Osteoporose en Metabole Botzieken“ van het Universitaire Ziekenhuis te Gent werden twee grootschalige populatiestudies gestart om enkele van de resterende vragen te kunnen beantwoorden.
Een eerste studie betreft de FAMOS Studie (FAMily Osteoporosis Study). Deze omvat drie-generatie familie stambomen uitgaande van mannen met idiopathische osteoporose.

De tweede studie is de SIBLOS Studie (SIBLing Osteoporosis Study). In deze studie werden broer-paren op de leeftijd van piekbotmassa gerecruteerd. Hierbij werd uitgegaan van een populatie benadering in verschillende gemeenten rondom Gent.

Curriculum Vitae :

GOEMAERE Stefan
Place and date of birth: Gent, December 29 1960
Address: Mariakerksesteenweg 58, 9031 Drongen
Telephone: +32 9 236 63 81
Mobile: +32 475 32 91 25
Inscription number University Ghent: 10.7453

Education

- Humanities : St. Vincentius college Eeklo (1972-1978) (Scientific direction A)
- Univervity studies:
  - Medical Doctor at Ghent Univesity (1978-1985)
  - Rheumatology at Ghent University (1985-1991) (Dpt. of Rheumatology Ghent – Prof. E.M. Veys)

Additional certificates

- Educational Commission for Foreign Medical Graduates (ECFMG):
  Basic Medical Science: 22 July 1986; Clinical Science Component: 20 July 1987
- Certified course on radioprotection for DXA (23/04/2004)
- IOF/ISCD certified course on bone densitometry (24-25/04/2004)
- Good Clinical Practice (GCP) experience and training during clinical trial meetings since 1991

Memberships

- Board member of "Belgian Bone Club"
- Member of the American Society for Bone and Mineral Research (ASBMR)
- Member of the international Bone and Mineral Society (IBMS)
- Member of "Koninklijke Belgische Vereniging van Reumatologie"
- Member of the International Society of Clinical Densitometry (ISCD)
- Member of the European Calcified Tissue Society (ECTS)
- Member of International Osteoporosis Foundation (IOF): BBC representant in the Committee of National Societies (CNS) and fracture working group of the Committee of Scientic Advisors.
- Member of the European Union Osteoporosis Consultation Panel (of IOF).

Present function

- Full time rheumatologist in the Department of Rheumatology and Endocrinology (University Hospital Ghent)
- Deputy director of the "Unit of Osteoporosis and Metabolic Bone Diseases" (Ghent University Hospital)

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THESIS, EDITOR OR CONTRIBUTOR BOOK (B2 PUBLICATIES)

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2. GEUSENS PPMM, LAAN RFJM, LEMS WF, **GOEMAERE S**. Metabole botziekten. In : Reumatologie en klinische immunologie.Eds. Bijlsma JWJ, Geusens PPMM, Tak PP. Bohn Stafleu Van Loghum, Houten,350-375,2004

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SCIENTIFIC PRIZES

- Wetenschappelijk prijs van Upjohn '94 uitgereikt door het NFWO met als titel:
  "Hormonale en klinische veroudering bij de man : determinanten en klinische relevantie van verminderde androgeen- en groeihormoonsecretie". J.M. Kaufman, S. Goemaere

- Prijs Prof. Dr. G. Verdonk (periode 2000-2002) uitgereikt door de Koninklijke Academie voor Geneeskunde van België met als titel:
  "Hormonal and genetic determinants of bone loss in community-dwelling elderly men: a longitudinal population study". S. Goemaere, I. Van Pottelbergh, J.M. Kaufman. Verhandelingen van de Koninklijke Academie voor Geneeskunde van België ; LXVI nr 2, blz 81-95.

List of abbreviations :

25(OH)D :	25 hydroxyvitamin D
$1,25(OH)_2D$:	1, 25 di-hydroxyvitamin D
aBMD :	Areal bone mineral density
Ac.F :	Activation frequency
ADT :	Androgen deprivation therapy
ALP :	Alkaline phosphatase
AP-1 :	Transcription factor AP-1
ATF4 :	Activating transcription factor 4.
ATP :	Adenosine tri-phosphate
AUC :	Area under the curve
BA :	Bone area
$BioE_2$:	Bioavailable estradiol
BioT :	Bioavailable testosterone
BM :	Bone marrow
BMC :	Bone Mineral Content
BMD :	Bone Mineral Density
BMP :	Bone morphogenic proteins
BMS :	Bone Material Strength
BsAP :	Bone specific alkaline phosphatase
BSU :	Basic Structural Unit
Brittleness :	biomechanical term
BTM :	Bone turnover marker
BUA :	Broadband ultrasound attenuation
CaCN :	Calcineurin
CamKII :	Ca^{2+}-calmodulin dependent kinase II
CAN :	Cerberus and Dan
CART :	Cocaine- and amphetamine-regulated transcript
CaSR :	Calcium-sensing receptor
C-fos :	Transciptional factor
CIZ :	Zinc finger protein 384
c-jun :	Transcription factor
CNS :	Central nervous system
CRD :	Cystine rich domain
CREB :	cAMP response binding element
CS :	Chondroitine sulfate
CT :	Computed tomography
CTX :	C-terminal type I collagen peptides
CV :	Coefficient of variation
CycD1 :	Cycline D1
CYP :	Cytochrome P
Dan :	Differential screening-selected gene aberrative in neuroblastoma.
DBM :	Degree of Bone Mineralization
DHEA(S) :	Dehydroepiandrosterone (sulphate)
Dkk :	Dickkopf
DNA :	Desoxyribonucleinic acid
DPD :	Desoxypyridinoline
DS :	Dermatan sulfate
Ductility :	Ability of a material to deform plastically without breaking
DXA :	Dual X-ray absorptiometry
E_1:	Estrone
E_2:	17β-estradiol
EDS:	Ehlers-Danlos syndrom
Elk-1 :	Transcription factor
ERE :	Estrogen response element

ERK :	Extra-cellular signal-regulated kinase
ESP :	European Standard Phantom
FE_2 :	Free estradiol
FEA :	Finite element analysis
FGF23 :	Fibroblast growth factor 23
FGFR1c :	Fibroblast growth factor receptor 1c
FSTL :	Follistatin like
Fra-1 :	Fos-related antigen-1
FSH :	Follicle stimulating hormone
FT :	Free testosterone
FZ :	Frizzled (Fz)
FRP :	Frizzled-related protein
GAG :	Glycosaminoglycans
GC:	Glucocorticoid
GDF :	Growth and differentiation factor
GDS :	Gut-derived serotonin
GH :	Growth hormone
GHR :	Growth hormone receptor
GHRH :	Growth hormone releasing hormone
GIOP :	Glucocorticoid-induced osteoporosis
GPI :	Glycosylphophatodylinositol
Gpc :	Glypican
GSK3B :	Glycogeen synthase kinase 3β
GWA :	Genome wide association
HBM :	High bone mass
HP :	Heparin
HPLC :	High performance liquid chromatography
HR :	Hazard ratio
HRE :	Hormone response element
HS :	Heparan sulfate
HSA :	Hip structure analysis
HSD :	Hydroxysteroid dehydrogenase
HSPG :	Heparan sulfate proteoglycan
Htr1B :	5-hydroxytryptamine (seretonine) receptor 1B
ICTP :	type I collagen cross-linked telopeptide
IGF :	Insuline-like growth factor
IGFBP :	Insuline-like growth factor binding protein
IGF-IR :	Insuline-like growth factor I receptor
IL-6 :	Interleukin 6
IOF :	International osteoporosis foundation
rhGH :	Recombinant human growth hormone
JNK :	c-Jun N-terminal kinase
Krm :	Kremen
KS :	Keratan sulfate
LH :	Luteinizing hormone
LRP :	Low density lipoprotein receptor related protein
LSC :	Least significant change
MAP :	Mitogen activated protein kinases
M-CSF :	Macrophage colony stimulating factor
MDP :	Methylene diphosphonate
MEK :	Meiosis-specific serine/threonine protein kinase
MES :	Minimum effective strain
MRI :	Magnetic Resonance Imaging
NF-κB :	nuclear factor-κB
NGS :	Next-generation sequencing
NTX :	N-terminal type I collagen peptides
OC:	Osteocalcin
OI:	Osteogenesis imperfecta
OR :	Odds ratio

OS :	Oxidative stress
OST-PTP :	Osteocalcin tyrosine phosphatase
OPG :	Osteoprotegerin
OPN	Osteopontin
OPPG :	Osteoporosis pseudoglioma
OPT :	Osteopetrosis
OSX :	Osterix
PBM :	Peak bone mass
PCP :	Planar cell polarity
PBL :	Peripheral blood leucocytes
PG :	Proteoglycan
PI3K :	Phosphatidyl inositol-3kinase
PICP :	Procollagen type I C-terminal propeptide
PINP :	Procollagen type I N-terminal propeptide
PTH :	Parathormone
PTHrP:	Parathormone related peptide
PYD :	Pyridinoline
QCT :	Quantitative computed tomography
QUS :	Quantitative ultrasound
RANK :	Receptor activator of nuclear factor-κB
RANKL :	Receptor activator of nuclear factor-κB ligand
ROC :	Receiver operator charateristic
ROS:	Reactive oxygen species
RR :	Relative risk
RUNX2 :	Runt related transcription factor 2
SD :	Standard deviation
sFRP :	Secreted frizzled-related protein
SHBG :	Sex hormone binding globuline
SLRP :	Small leucine-rich repeat proteoglycan
SNP :	single nucleotide polymorphism
SOD :	Superoxide dismutases
SOS:	Speed of sound
Stiffness :	biomechanical term
T :	Testosterone
TGF-β :	Transforming growth factor-β
TL :	Telomere length
TNF :	Tumor necrosis factor
TPH1 :	Tryptophan hydroxylase 1
TRACP :	Tartrate resistant acid phosphatase
TRF :	Telomere restriction fragment
unOC :	Undercarboxylated ostecalcin
vBMD :	Volumetric bone mineral density
VDR :	Vitamin D receptor
VFA :	Vertebral fracture assessement
Wg :	Wingless
WHO :	World Health Organisation
Wif-1 :	Wnt inhibitory factor 1
Wnt :	Wingless-related integration site