INTRODUCTION

1.1 Bone: An Architectural Masterpiece

Bone is a vital, dynamic and complex connective tissue that provides form/shape to the body, supporting its weight. Bone has multiple functions in vertebrates, including protection of vital organs and the environment and niches required for haematopoiesis, mechanical and structural support for muscles and joints, and a mineral reservoir that is essential for calcium homeostasis, and of growth factors stored in the matrix [Lacey et al., 2012; Rouhi, 2012; Boyce and Xing, 2008; Morgan et al., 2008].

1.1.1 Bone Structure

Bone is not a uniformly solid material, but rather has some spaces between its hard elements.

(a) Compact (Cortical) bone

(b) Trabecular (Cancellous or Spongy) bone

Figure I: Compact Bone and Spongy Bone, Source: U.S. National Cancer Institute’s surveillance, Epidemiology and End Results (SEER) (http://training.seer.cancer.gov/index.html).


1.1.1.1 Compact (Cortical) Bone

Cortical bone makes up the hard, dense outer shell of the skeleton [Riggs et al., 2002]. Cortical bone constitutes approximately 80% of the skeletal mass. It has minimal gaps and spaces. Its porosity is 5–30% [Hall, 2007].

1.1.1.2 Trabecular (Cancellous or Spongy) Bone

Trabecular bone fills the ends of the limb bones and the vertebral bodies in the spine, the sites of most osteoporotic fractures [Riggs et al., 2002]. Trabecular bone constitutes approximately 20% of the skeletal mass. Filling the interior of the bone is the trabecular bone tissue which is composed of a network of rod- and plate-like elements that make the overall organ lighter and allow room for blood vessels and marrow. It has nearly ten times the surface area of compact bone. Its porosity is 30–90% [Hall, 2007].

1.1.2 Bone Major Functions

Bone has two major functions:

(a) Provision of mechanical integrity for locomotion and protection, and

(b) Involvement in the metabolic pathways associated with mineral homeostasis.

The structure and composition of this tissue reflect a balance between these two major functions. Besides this, bone is also the primary site of hematopoiesis. There is a complex interplay between the bone organ system and the immune system [Bab and Einhorn, 1994; Horowitz and Jilka, 1992; Wein et al., 2005; Boyce and Xing, 2007; Morgan et al., 2008].

1.1.3 The Structure-Function Relationship

Galileo, in 1638, proposed an early hypothesis about the dependence of the structure and form of bones, and the mechanical loads they carry [Ascenzi, 1993; Rouhi, 2012]. Wolff in 1892 described this concept in a semi quantitative manner [Wolff, 1986; Rouhi, 2012]. The structure-function relationships observed in bone under normal physiological conditions, coupled with its role in maintaining mineral homeostasis,
strongly suggest that it is an organ of optimum structural design [Rouhi, 2012]. In maintaining the structure–function relationship, bone tissue is constantly being broken down and rebuilt in a process called remodeling [Rucci, 2008]. Bone has the potential to adapt its architecture, shape, and mechanical properties via a continuous process termed adaptation in response to altered loading conditions [Burr et al., 2002; Forwood and Turner, 1995; Hsieh and Turner, 2001]. The regulation of bone mass in mammals is governed by a complex interplay between bone-forming cells termed osteoblasts (OBs) and bone-resorbing cells termed osteoclasts (OCs), and is guided physiologically by a diverse set of hormones, cytokines and growth factors. The balance between these processes changes over time, causing an elevated risk of fractures with age [Lacey et al., 2012]. With age, remodeling tends to result in a negative bone balance due to decrease in the number of osteoblasts, in that at each remodeling site slightly less bone is deposited than is resorbed. This negative balance leads to osteopenia and osteoporosis (OP), thus predisposing the bone to fracture during even minimal trauma [D’ippolito et al., 1999; Morgan et al., 2008].

1.2 Bone Composition

Bone is a composite material consisting of two phases:

(a) Inorganic phase.

(b) Organic phase.

By weight, approximately 60-70% of the tissue is inorganic matter, 8–10% is water, and the remainder is organic matter [Gong et al., 1964]. By volume, these proportions are approximately 40%, 25%, and 35%, respectively [Morgan et al., 2008].

![Bone Composition Diagram]

Figure II: Bone Composition, Source: Atlas of Postmenopausal Osteoporosis, Third Edition.
Table 1: Bone Composition

<table>
<thead>
<tr>
<th>Components</th>
<th>By Weight (%)</th>
<th>By Volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic Matter</td>
<td>60-70%</td>
<td>40%</td>
</tr>
<tr>
<td>Water</td>
<td>8-10%</td>
<td>25%</td>
</tr>
<tr>
<td>Organic Matter</td>
<td>22-32%</td>
<td>35%</td>
</tr>
</tbody>
</table>

1.2.1 Organic Phase

The organic phase is composed of three components [Clarke, 2008].

(a) Type I collagen,

(b) A variety of non-collagenous proteins (NCPs),

(c) Cells.

Type I collagen and variety of noncollagenous proteins constitute 98% by weight, of organic phase and cells make up the remaining 2% of this phase [Einhorn, 1994; Morgan et al., 2008].

1.2.1.1 Role of the Organic Phase

The organic phase of bone plays a wide variety of roles:

(a) It influences the structure of the bone.

(b) It also influences the mechanical and biochemical properties of the tissue also.

(c) Growth factors, cytokines and extra cellular matrix (ECM) proteins such as osteonectin, osteopontin, bone sialoprotein, osteocalcin, proteoglycans, and other phosphoproteins and proteolipids, make major contributions to biologic function of bone [Morgan et al., 2008].
1.2.1.2 Type I Collagen

Properties

(a) It is a ubiquitous protein of extremely low solubility.

(b) The major structural component of the bone matrix.

(c) Its molecule consists of three polypeptide chains composed of approximately 1000 amino acids each.

(d) These chains take the form of a triple helix of two identical 1(I) chains and one unique 2(I) chain cross-linked by hydrogen bonding between hydroxyproline and other charged residues.

(e) This produces a very rigid linear molecule that is approximately 300 nm in length.

(f) Each molecule is aligned with the next in a parallel fashion and in a quarter-staggered array to produce a collagen fibril.

(g) The collagen fibrils are then grouped in bundles to form the collagen fiber.

(h) Within the collagen fibril, gaps known as “hole zones” are present between the ends of the molecules.

(i) Pores exist between the sides of parallel molecules.

(j) Noncollagenous proteins or mineral deposits can be found within these spaces, and mineralization of the matrix is thought to be initiated in the hole zones [Morgan et al., 2008].
Figure III (a) The scanning electron image shows bone collagen fibrils in both longitudinal and cross sections. (b) A back-scattered electron image of the regular patterns of collagen in layers in normal, or lamellar, bone. Source: Atlas of Postmenopausal Osteoporosis, Third Edition.

1.2.1.3 Non-Collagenous Proteins (NCPs) of the Extracellular Matrix

There are three types of NCPs of ECM found:

(a) Structural matrix proteins
(b) Enzymatic Matrix Modifiers
(c) Bone Morphogens

1.2.1.3.1 Structural Matrix Proteins

There are six Structural Matrix Proteins found in the organic phase of bone.

(a) Osteocalcin (OCN)
(b) Osteopontin (OPN)
(c) Bone Sialoprotein (BSP)
(d) Decorin
1.2.1.3.1.1 Osteocalcin (OCN)

Properties

(a) It is one of the most abundant noncollagenous proteins in bone [Morgan et al., 2008].

(b) It has been recognised as a bone-derived hormone to regulate energy metabolism [Fernández-Real and Ricart, 2011; Li et al., 2011; Zivna et al., 2013].

(c) It is produced by active osteoblasts [Duncan et al., 1998], and restricted to the osteoblast lineage only.

(d) It is also called bone-carboxyglutamic acid-containing protein (bone Gla protein) [Morgan et al., 2008].

(e) It is a small protein (molecular weight is approximately 5.8 kDa) having three glutamic acid residues carboxylated as a result of a vitamin K dependent, post-translational modification.

(f) The carboxylation of these residues confers on this protein calcium and mineral binding properties.

(g) It accounts for 10–20% of the noncollagenous protein content [Morgan et al., 2008].

(h) It is closely associated with the mineral phase.

(i) This bone-specific protein may regulate activities of osteoclasts and osteoclast precursors.
Introduction

(j) Through characterization of the phenotype of osteocalcin-deficient mice, it was also found that osteocalcin has an important role in inhibiting bone formation and in mineral maturation [Boskey et al., 1998].

1.2.1.3.1.2 Osteopontin (OPN)

Properties

(a) Osteopontin is expressed by a variety of cells [Li et al., 2011].

(b) It is a member of the small integrin-binding ligand N-linked glycoprotein family.

(c) It is highly expressed in bone and inflammatory tissue.

(d) It contains an arginine–glycine–aspartic acid (RGD) sequence.

(e) These amino acid sequences are the characteristic of cell-binding proteins.

(f) These are recognized by a family of cell membrane proteins known as integrins.

(g) The integrins span the cell membrane and provide a link between the extracellular matrix and the cytoskeleton of the cell.

(h) Integrins on osteoblasts, osteoclasts, and fibroblasts provide a means for anchoring these cells to the extracellular matrix.

(i) Once anchored, the cells are then enabled to express their phenotype and conduct the types of activities that characterize their functions [Ruoslahti, 1991].

(j) Thus, it supports osteoblast attachment to bone.

(k) It binds and activates matrix metalloproteinase-3 (MMP-3) [Morgan et al., 2008].

1.2.1.3.1.3 Bone Sialoprotein (BSP)

Properties

(a) BSP is a marker of terminally differentiated osteoblasts [Li et al., 2011].

(b) BSP is selectively expressed in mineralizing tissues and, especially, at sites of de novo bone formation [Chen et al., 1994; Li et al., 2011].

(c) It is a member of the sibling family.

(d) It may initiate mineralization.
It supports cell attachment.

It binds $\text{Ca}^{+2}$ with a high affinity.

It binds and activates matrix metalloproteinase-2 (MMP-2) [Morgan et al., 2008].

1.2.1.3.1.4 Decorin

Properties

(a) Decorin is also known as chondroitin sulphate proteoglycan I.

(b) It regulates collagen fibrillogenesis and transforming growth factor $\beta 1$ (TGF$\beta 1$) activity.

(c) It binds to fibrinogen [Morgan et al., 2008].

1.2.1.3.1.5 Biglycan

Properties

(a) Biglycan is also known as chondroitin sulphate proteoglycan II.

(b) It is involved in the regulation of fibrillogenesis.

(c) It modulates bone morphogenic protein-2 (BMP-2) induced osteogenesis [Morgan et al., 2008].

1.2.1.3.1.6 Osteonectin (ON)

Properties

(a) Osteonectin is expressed in a variety of connective tissues.

(b) It has strong affinity for $\text{Ca}^{+2}$.

(c) It may play a role in matrix mineralization [Morgan et al., 2008].

1.2.1.3.2 Enzymatic Matrix Modifiers

There are four Enzymatic Matrix Modifiers

(a) Matrix metalloproteinases (MMPs)

(b) Tissue inhibitors of MMPs (TIMPs)

(c) Lysyl Oxidase

(d) Stromelysin
1.2.1.3.2.1 Matrix metalloproteinases (MMPs)

Properties

(a) These include collagenases (MMP-1 and -13) and gelatinases (MMP-2 and -9).

(b) MMPs are required for collagen degradation.

(c) Most of them are expressed in mature chondrocytes and osteoblasts [Morgan et al., 2008].

1.2.1.3.2.2 Tissue inhibitors of MMPs (TIMPs)

These are the inhibitors of MMP activity [Morgan et al., 2008].

1.2.1.3.2.3 Lysyl Oxidase

Lysyl Oxidase is a copper-dependent extracellular enzyme that catalyzes oxidative deamination of elastin and collagen precursors leading to the formation of a mature ECM [Morgan et al., 2008].

1.2.1.3.2.4 Stromelysin

Properties

(a) Stromelysin is a member of the MMP family (MMP-3).

(b) It degrades most components of the ECM.

(c) It activates other MMPs [Morgan et al., 2008].

1.2.1.3.3 Bone Morphogens

There are three types of Bone Morphogens.

(a) TGFβ Super Family

(b) Fibroblast growth factors (FGFs)

(c) Platelet-derived growth factors (PDGFs)

1.2.1.3.3.1 Transforming growth factor β (TGFβ) super family of morphogens

Properties
1.2.1.3.3.2 Fibroblast growth factors (FGFs)

**Properties**

(a) FGFs 1 and 2 have angiogenic properties.

(b) FGFs promote cellular proliferation [Morgan et al., 2008].

1.2.1.3.3.3 Platelet-derived growth factors (PDGFs)

**Properties**

(a) These exist in three forms (AA, AB and BB).

(b) PDGFs are associated with mesenchymal cell chemotaxis and proliferation [Morgan et al., 2008].

1.2.2 Inorganic Phase

**Properties**

(a) It is an impure form of hydroxyapatite (Ca_{10}[PO_{4}]_{6}[OH]_{2}).

(b) It is a naturally occurring calcium phosphate.

(c) The small plate-shaped apatite crystals contain impurities, most notably carbonate in place of the phosphate groups.

(d) The concentration of carbonate (4–6%) makes bone mineral similar to a carbonate apatite known as dahllite.

(e) Potassium, magnesium, strontium, and sodium in place of the calcium ions and chloride and fluoride in place of the hydroxyl groups are also found [McConnell, 1962].
These impurities reduce the crystallinity of the apatite [Ou-Yang et al., 2001], and in doing so may alter certain properties such as solubility [Kaplan et al., 1994].

The solubility of bone mineral is critical for mineral homeostasis and bone adaptation.

In Paget’s disease [Siris, 1998] and diabetes [Einhorn et al., 1988], crystal size is reported to be decreased.

In osteopetrotic individuals [Boskey and Marks, 1985] and with bisphosphonate treatment [Fratzl et al., 1996; Morgan et al., 2008], the crystal size is found to be increased.

1.3 Bone Cellular Components

1.3.1 Bone Cell Types

There are three cell types typically associated with bone homeostasis.

(a) Osteoblasts (“bone builders”) 
(b) Osteocytes (“bone maintainers”, “bone controllers”) 
(c) Osteoclasts (“bone resorbers”, “bone breakers”, “bone carvers”)

These three cell types are derived from two separate stem cell lineages—the mesenchymal lineage and the hematopoietic lineage [Morgan et al., 2008].

1.3.1.1 Mesenchymal Lineage Cells

1.3.1.1.1 Osteoblast

Properties

(a) Osteoblasts (“bone builders”) commonly called bone-forming cells which derived from the osteoblast progenitor cells, participate in mineralization and are unable to multiply [Clarke, 2008].

(b) These are the specialized fibroblasts that in addition to fibroblastic products, express BSP and osteocalcin [Salentijn, 2007].
(c) Both embryonic and postnatal bone formation is carried out by the mesenchymal lineage osteoblast.

Figure IV: Osteoblasts (blue) rimming a bony spicule (pink-on diagonal of image), Source:http://en.wikipedia.org/wiki/File:Bone_hypercalcemia_-_cropped_-_very_high_mag.jpg

(d) Osteoblasts produce the protein matrix of bone made up of type I collagen and several noncollagenous proteins.

(e) This protein matrix is referred to as the osteoid which creates a template for mineralization and production of the mature bone.

(f) Zinc, copper and sodium are some of the minerals required in this process [D’ippolito et al., 1999].

(g) Osteoblasts also assist with the initiation of bone resorption by secreting factors macrophage-colony stimulating factor (M-CSF) [Buckley et al., 2005], receptor activator for nuclear factor kappa b ligand (RANKL) and osteoprotegerin (OPG) that recruit and promote the differentiation of monocytic lineage cells into mature osteoclasts and also by producing neutral proteases that degrade the osteoid and prepare the bone surface for osteoclast-mediated remodeling [Wada et al., 2006].
Mesenchymal stem cells (MSCs) are the pluripotent cells that can differentiate into a variety of cell types including myoblasts, adipocytes, chondrocytes, osteoblasts, and osteocytes in bone marrow [Kamon et al., 2010; Rayalam et al., 2011].

Osteoblasts differentiate from the mesenchymal stem cell under the stimulation of BMPs [Katagiri and Takahashi, 2002].

The specific lineage selection of an individual mesenchymal stem cell involves a number of coordinated lineage selection steps and the actions of a number of transcriptional regulators, in particular the BMPs [Agata et al., 2007].

BMP4 and FGF2 have been experimentally shown to increase osteoblast differentiation [Lee et al., 2013].

Figure V: Osteoblast Organization and Mineralization of Bone, Source:http://en.wikipedia.org/wiki/File:Osteoblast_Organization.jpg
1.3.1.1.1 Osteoblast Formation and Differentiation

[Kobayashi and Kronenberg, 2005] reported that there are two transcription factors which have been demonstrated to be required for osteoblast formation and differentiation:

(a) RUNX2

(b) Osterix

1.3.1.1.1.1 Runx-Related Transcription Factor 2 (RUNX2)

Properties

(a) RUNX2 is best known as an essential master transcription factor in osteoblast differentiation and bone development [Li et al., 2011; Ferrari et al., 2013; Yoon et al., 2013].

(b) RUNX2 also known as core-binding factor subunit alpha-1 (CBF-α-1) is a protein that in humans is encoded by the Runx2 gene.

(c) RUNX2 is a member of the runt homology domain transcription factors [Morgan et al., 2008].

(d) It acts as a scaffolding protein organizing nuclear complexes at discrete sites on the nuclear matrix associated with active gene transcription.

(e) The differentiation of osteoblasts is led by the induction of expression of alkaline phosphatase (ALP) or bone matrix proteins such as type I collagen, OCN and OPN, under the action of the transcription factors Runx2 and Osterix [Nakashima et al., 2002; Komori, 2005].

(f) The repression of the differentiation factor to muscle cell and adipocyte also leads to the differentiation to osteoblasts [Nakashima et al., 2002; Komori, 2005].

(g) Thus, RUNX2 is indispensable for mature osteoblast differentiation and function [Meyer et al., 2014].
Differentiation leads to a restricted RUNX2 cistrome that correlates with changes in gene expression [Meyer et al., 2014].

1.3.1.1.1.1.2 Osterix (Osx)

Properties

(a) Osterix is a novel zinc finger–containing transcription factor that is essential for osteoblast differentiation and bone formation [Cao et al., 2005; Li et al., 2011].

(b) The Osterix knockouts also lack embryonic bone formation and osteoblast differentiation [Nakashima et al., 2002].

(c) Osterix functions downstream of RUNX2 activity as RUNX2-/- cells express no Osterix.

(d) In the Osterix knockout mice, the pool of RUNX2-expressing pre-osteoblasts express several genes associated with chondrogenesis, suggesting Osterix plays a role in stabilizing osteogenic commitment and osteoblast maturation.

(e) The relative expression and activity of RUNX2 and Osterix are regulated by the local microenvironment and the locally produced morphogens to which the cells are exposed.

(f) Growth factors including members of insulin-like growth factors (IGFs), FGFs, TGF-β, BMPs, and Wnts have all been demonstrated to play important roles in regulating embryonic osteoblast differentiation.

(g) Once osteoprogenitor start to differentiate into osteoblasts, they begin to express a range of genetic markers including collagen 1 (Col1), osterix, BSP, M-CSF, ALP, OC, OPN and ON [Ringe et al., 2008; Szulc et al., 2005].

(h) These cells are also characterized by the expression of different markers such as RANKL, OPG [Wauquier et al., 2009; Wada et al., 2006].
1.3.1.1.2 Regulation of Osteoblast Differentiation

Osteoblast differentiation and bone formation are regulated by transcriptional and post-transcriptional mechanisms [Chen et al., 2014]. More recently, micro RNAs (miRNAs) were identified as novel key regulators of human stromal (skeletal, mesenchymal) stem cells (hMSC) differentiation [Chen et al., 2014]. Osteoblast differentiation from MSCs is also tightly regulated. An insufficiency of peroxisome proliferator activated receptor γ (PPARγ), promotes osteoblast formation. PPARγ, a key transcription factor, is considered a master regulator of adipogenesis and is implicated in lipid metabolism [Pei and Tontonoz, 2004]. Deletion of PPARγ resulted in an increased expression of key molecules for osteoblastic differentiation like Runx2 and osterix. Wnt signaling also diverts MSCs towards osteogenic lineage. While osteoblast precursors enhance bone resorption by stimulating RANKL-induced osteoclast activation, mature osteoblasts block RANKL-induced osteoclastogenesis by increasing osteoprotegerin expression [Goldring and Goldring, 2007]. The activation of sirtuin 1(Sirt1) promoted osteoblast differentiation in bone marrow stromal cells and MSCs [Backesjo et al., 2009; Backesjo et al., 2006].

![Adipocytes and osteoblast life cycle](image)

Figure VI: Adipocytes and osteoblast life cycle [Rayalam et al., 2011].
1.3.1.1.3 Morphology

Properties

(a) An active osteoblast has a prominent Golgi apparatus that appears histologically as a clear zone adjacent to the nucleus.

(b) The nucleus is spherical and large.

(c) The cytoplasm of osteoblasts is basophilic due to the presence of a large amount of rough endoplasmic reticulum (RER) as revealed by haematoxylin and eosin staining (H&E staining).

(d) Active osteoblasts synthesize and stain positively for Col1 and ALP [Clover et al., 1992].

1.3.1.1.4 Osteoblasts-Osteocytes Relationship

During bone formation and remodeling, osteoblasts get embedded into the matrix they deposit and differentiate to osteocytes [Kerschnitzki et al., 2013]. They cease to generate osteoid and mineralized matrix, and instead act in a paracrine manner on active osteoblasts. They are believed to respond to mechanosensory stimuli [Ehrlich and Lanyon, 2002; You et al., 2000].

1.3.1.1.2 Osteocyte

Properties

(a) Osteocytes (“bone maintainers”, “bone controllers”) are mature osteoblast which no longer secretes matrix, participates in nutrient/waste exchange via blood and unable to divide [Clarke, 2008].

(b) Osteocytes are a type of osteoblast and thus differentiate from the same mesenchymal lineage under the regulation of the same transcription factors discussed previously [Tate et al., 2004; Franz-Odendaal et al., 2006].

(c) Osteocytes, escape apoptosis, reduce their production of matrix molecules, and eventually end up encapsulated in the bone matrix.
(d) In the bone they are characterized by their long processes that extend through the lacunocanalicular system of the bone.

(e) Osteocytes are in fact the most abundant cellular component of mammalian bones, making up 95% of all bone cells.

(f) Osteocytes must survive for decades within the bone matrix, making them one of the longest lived cells in the body [Dallas et al., 2013].

(g) Osteocytes are long lived, relative to the other bone cells, with estimates running as high as 25 years, as compared to osteoblasts, which are estimated in humans to live approximately an average of 3 months [Tate et al., 2004].

(h) These cells form a dense network throughout the entire bone material [Kerschnitzki et al., 2013].

1.3.1.2.1 Functions

(a) Osteocytes are known to orchestrate bone remodeling [Kerschnitzki et al., 2013].

(b) Osteocytes encased within mineralized bone matrix are actually multifunctional cells with many key regulatory roles in bone and mineral homeostasis [Dallas et al., 2013].

(c) Osteocytes are mechanosensory cells that coordinate adaptive responses of the skeleton to mechanical loading, and also serve as a manager of the bone’s reservoir of calcium.

(d) The pericellular matrix (PCM), a thin coating surrounding nearly all mammalian cells, plays a critical role in many cell-surface phenomena [Wang et al., 2014].

(e) In osteocytes, the PCM is believed to control both "outside-in" (mechanosensing) and "inside-out" (signaling molecule transport) processes [Wang et al., 2014].
(f) Osteocytes create an interconnected network in bone allowing for intercellular communications between both neighboring osteocytes and the surface-lining osteoblasts.

(g) The osteocyte network provides access to a huge mineral reservoir in bone due to its dense organization [Kerschnitzki et al., 2013].

Figure VII: Structure of osteocyte, 

(h) This interconnection between osteocytes allows for the transmission of mechanical and chemical signals across the network through direct transmission of mechanical forces either through the triggering of integrin force receptors, changes in membrane conformation, chemical signals via the gap junctions, or secreted factors that travel through the extracellular fluid of the lacunocanalicular system [Tate et al., 2004].

(i) Osteocytes interact with their mineralized vicinity and thus, participate in bone mineral homeostasis [Kerschnitzki et al., 2013].
1.3.1.2 The Hematopoietic Cell Lineage

1.3.1.2.1 Osteoclasts (OCs)

Properties

(a) Osteoclasts ("bone resorbers", "bone breakers", "bone carvers") were discovered by Kolliker in 1873 [Nijweidi and Feyen, 1986].

(b) Osteoclasts are capable of resorbing mineralized bone and excessive bone resorption by osteoclasts causes bone loss related diseases [Moon et al., 2012].

(c) Osteoclastic bone resorption depends upon the cell's ability to organize its cytoskeleton [Fukunaga et al., 2014].

(d) Osteoclasts are bone-resorbing cells derived from hematopoietic precursors of the monocyte-macrophage lineage and participate in bone resorption [Kanzaki et al., 2014; Zaidi et al., 2003; Quinn and Gillespie, 2005; Fattore et al., 2008; Clarke, 2008; Wauquier et al., 2009; Lampropoulos et al., 2012].

(e) They are formed by the fusion of precursors that are derived from the pluripotent hematopoietic stem cell [Faust et al., 1999; Netter, 1987; Suda et al., 1992] and circulate in the monocyte fraction [Udagawa et al., 1990; Fujikawa et al., 1996].

(f) Osteoclasts are unique multinucleated cells which show the ability to destroy the bone extracellular matrix through the dissolution of hydroxyapatite and the degradation of the organic matrix components [Teitelbaum, 2007; Suda et al., 1992; Wada et al., 2006; Wauquier et al., 2009; Ha et al., 2013].

(g) To do so, osteoclasts must undergo a series of changes which imply massive cytoskeletal remodeling, polarization of plasma membrane, redistribution of transporters and organelles, as well as prominent endosomal trafficking [Teitelbaum, 2007].

(h) Osteoclasts are characterized by high expression of tartrate resistant acid phosphatase (TRAP), cathepsin K (CTSK), receptor activator for nuclear
factor kappa B (RANK) and calcitonin receptor (CTR) expression [Wauquier et al., 2009].

(i) They are post-mitotic cells with a pre-definite life span which varies among species from days to weeks [Tanaka et al., 2006].

(j) Osteoclast number must be kept tightly controlled to maintain physiological remodeling and prevent excess of bone resorption leading to pathological bone loss [Bruzzaniti and Baron, 2006].

(k) Inflammatory cytokines play a major role in osteoclastogenesis, leading to the bone resorption that is frequently associated with cancers and other diseases [Bharti et al., 2004].

(l) Besides the well known RANK, RANKL and OPG axis, a variety of factors tightly regulate osteoclast formation, adhesion, polarization, motility, resorbing activity and life span, maintaining bone resorption within physiological ranges [Fattore et al., 2008].

(m) Nuclear receptors, cell surface receptors, integrin receptors and cell death receptors work together to control osteoclast activity and prevent both reduced and increased bone resorption [Fattore et al., 2008].

(n) Osteoclasts play a role in balancing calcium homeostasis with skeletal remodeling.

(o) These cells are found at the apex of the classical “cutting cones” in cortical bone and in the resorptive cavities known as Howship’s lacunae on trabecular bone surfaces undergoing active remodeling.

1.3.1.2.1.1 Morphology

Properties

(a) An osteoclast is a large cell (approximately 40 µm in diameter) containing around 15-20 closely packed oval-shaped nuclei in a cytoplasm with a homogeneous, "foamy" appearance.
(b) Foamy appearance is due to a high concentration of vesicles and vacuoles.

(c) These vacuoles are lysosomes filled with acid phosphatase.

(d) In osteoclast, Golgi complexes are found in large area of cytoplasm and RER are scattered. [Standring, 2005; Holtrop and King, 1977; Vaananen et al., 2000].

![Figure VIII: Osteoclast cell with multiple nuclei](http://upload.wikimedia.org/wikipedia/commons/1/1a/Osteoclast.jpg)

1.3.1.2.1.2 Ruffled border formation

At the active bone resorption site, the osteoclast forms a specialized cell membrane called ruffled border that touches the surface of the bone tissue [Netter, 1987].

**Properties**

(a) This ruffled border facilitates removal of the bony matrix.

(b) Ruffled border is a morphologic characteristic of an osteoclast that is actively resorbing bone.

(c) The ruffled border increases surface area interface for bone resorption.

Calcium and phosphate ions are the main components of the mineral portion of the matrix. These ions are absorbed into small vesicles, which move across the cell and
eventually are released into the extracellular fluid, thus increasing levels of the ions in
the blood [Morgan et al., 2008].

1.3.1.2.1.3 Bone Resorption Mechanism

An osteoclast is a member of the mononuclear phagocyte series and may be thought
of as a specialized type of macrophage [Quinn and Gillespie, 2005].

Steps

(a) In order to remove bone, newly formed osteoclasts become polarized.

(b) Then they form a ruffled membrane.

(c) Then adhere tightly to the bone matrix via an αvβ3 integrin mediated binding to
the bone surface to form the “sealing zone.”

(d) The osteoclast then secretes acid via H⁺-adenosine triphosphatases (ATPase)
and proteases including CTSK into this closed micro compartment along the
bone surface referred to as the hemi vacuole thereby removing the underlying
bone.

(e) H⁺-ATPase is used for hydroxyapatite dissolution.

(f) CTSK is used for matrix protein digestion.
By focusing the secretion of these acids and enzymes, osteoclasts are able to move along a bone surface or into a cutting cone slowly solubilising bone in a defined area without disrupting the surrounding local microenvironment [Morgan et al., 2008].

1.3.1.2.1.4 Osteoclasts Activation by ROS

Reactive oxygen species (ROS) act as intracellular signaling molecules in the regulation of receptor activator of nuclear factor-κB ligand (RANKL)-dependent osteoclast differentiation, but they also have cytotoxic effects that include peroxidation of lipids and oxidative damage to proteins and DNA [Kanzaki et al., 2014; He et al., 2010]. Osteoclasts have been shown to be activated by ROS [Bax et al., 1992; Garrett et al., 1990]. Several signals essential for osteoclasts are sensitive to activation by ROS, including Nuclear Factor-Kappa B (NF-κB), c-Jun N-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K) and p38 mitogen-activated protein kinase (p38 MAPK) [Paolicchi et al., 2002; Finkel and Holbrook, 2000]. Osteoclasts contain a nicotinamide adenine dinucleotide phosphate- reduced form (NADPH)
oxidase [Steinbeck et al., 1994], an enzyme that is capable of cytokine-regulated generation of ROS. ROS are also potent inducers in many cells of tumor necrosis factor-alpha (TNF-α) and other cytokines strongly implicated in the bone loss of estrogen deficiency. Thus, if estrogen increases oxidant defenses in bone, estrogen deficiency might directly or indirectly stimulate osteoclastic bone resorption.

1.3.1.2.1.5 Osteoclast Differentiation

During osteoclast differentiation, ROS act as secondary messengers on signal pathways [Moon et al., 2012]. The early differentiation stages of osteoclast formation depend on the transcription factor PU.1 which regulates the cell-surface receptor c-fms expression along with the transcription factor src [Zaidi et al., 2003; Quinn and Gillespie, 2005]. The cell-surface receptor c-fms is M-CSF receptor. M-CSF is also called colony-stimulating factor-1 (CSF-1). The expression of c-fms is a central component of early osteoclast formation as M-CSF responsiveness is required for both monocyte progenitor proliferation and the expression of RANK. Interleukin-1 (IL-1) and RANKL itself have been demonstrated to play a role in enhancing osteoclast activity. IL-1, IL-6, M-CSF, TNF-α, lipopolysaccharides (LPS) prolong the life span of an osteoclast [Zaidi et al., 2003; Quinn and Gillespie, 2005; Wada et al., 2006]. Osteoclast maturation and function primarily depend on RANKL-mediated induction of nuclear factor of activated T cells c1 (NFATc1), which is further activated via increased intracellular calcium ([Ca^{2+}]_i) oscillation [Kim et al., 2013].

1.3.1.2.1.6 Regulation

Osteoclasts are regulated by several hormones including parathormone or parathyroid hormone PTH [Raisz, 2005], calcitonin (CT) from the thyroid gland, and growth factor IL-6. Osteoclast activity is also mediated by the interaction of two molecules produced by osteoblasts, namely OPG and RANKL. These molecules also regulate differentiation of the osteoclast [Schoppet et al., 2002]. Vinculin (VCL), an actin-binding protein has been reported to regulate osteoclast function and participate in skeletal degradation [Fukunaga et al., 2014]. Recently, vinculin expression has been reported to regulate osteoclast function in a talin-dependent, αvβ3 integrin independent manner [Fukunaga et al., 2014].
1.4 Bone Homeostasis

Bone homeostasis involves two important processes as

(a) The constant remodeling process
(b) The constant rebuilding process

Figure X: Illustrated diagram of Bone Homeostasis, source [Redlich and Smolen, 2012].

This resultant process leads to the replacement of 4–10% of bone each year in humans. The bone formation side of the equation is carried out by the mesenchymal lineage-derived osteoblasts; the remodeling side of the homeostasis equation in bone is carried out by the hematopoietic lineage osteoclasts [Morgan et al., 2008]. Bone formation is related to osteoblastic proliferation, ALP activity, and osteocalcin and collage synthesis [Morgan et al., 2014]. Bone resorption is associated with osteoclast formation and differentiation, and TRAP [Morgan et al., 2014]. Usually, the amount of bone remains constant, as bone homeostasis is maintained through a balance
between osteoblastic bone formation and osteoclastic bone resorption [Yamaguchi et al., 2012], and the number and function of osteoclasts and osteoblasts are kept normal by the mutual interaction of these cells [Takahashi et al., 1988a; Jimi et al., 1996]. Bone loss is induced due to decreased osteoblastic bone formation and increased osteoclastic bone resorption with various pathologic states [Yamaguchi et al., 2012]. Circulating hormones together with locally produced cytokines and growth factors modulate the replication and differentiation of osteoclast and osteoblast progenitors [Jilka, 2003; Raisz, 2005].

### 1.4.1 Osteoblast-Osteoclast Coupling

Osteoblasts influence osteoclast formation and activity, and likewise osteoclasts influence osteoblast differentiation and activity. This coordination is referred to as “coupling” [Cao, 2011]. New bone formation occurs at bone resorption sites in each cycle of bone remodeling to maintain the micro architecture required for bone's mechanical properties.

#### 1.4.1.1 Cellular Communication Levels

There are three main communication levels in osteoblast-osteoclast coupling:

(a) In bone matrix, coupling of resorption and formation through TGF-β1 and IGF-1 [Negishi-Koga et al., 2011].

(b) Osteoclast-osteoblast communication through semaphorin-4D (Sema4D)-Plexin-B1 (PLXN-B1).

(c) RANK-RANKL mediates communication to induce differentiation of osteoclast progenitors.
1.4.1.2 Mechanism

The level of expression of the cytokines changes with the immature osteoblast producing the highest levels of M-CSF and RANKL during osteoblast differentiation.

Steps

(a) When an osteoblast begins to mature into a matrix-producing bone cell, it signals to local osteoclast precursors with RANKL to differentiate, thereby coupling the new bone formation with the recruitment of new osteoclasts for its subsequent remodeling.

(b) By coordinating osteoclast differentiation with osteoblast differentiation, the system stays in balance.

(c) Osteoclasts signal back to osteoblast progenitors through the release of BMPs and other growth factors that promote osteogenesis from the bone matrix as a
Introduction

part of the bone removal process completing the circle [Martin and Sims, 2005].

1.4.1.3 Significance

This coupling system has been evolutionarily adapted to provide a means of responding to more mechanical forces and systemic metabolic requirements. Bones adapt to the mechanical forces placed upon them. The interconnected osteocytes network is widely perceived to provide mechanosensory feedback that is communicated to the lining osteoblasts [Tate et al., 2004]. Intracellular responses to mechanical input can include increased cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP3), intracellular calcium, and activation of MAPK pathway [Rubin et al., 2006]. Bone remodeling plays an important role in systemic mineral homeostasis, with \( \text{Ca}^{2+} \) being the primary mineral stored in bone. Systemic \( \text{Ca}^{2+} \) levels are monitored by \( \text{Ca}^{2+} \) sensors in the parathyroid gland. As \( \text{Ca}^{2+} \) levels drop, the parathyroid releases PTH. Systemic PTH leads to increased remodeling and the release of \( \text{Ca}^{2+} \), bringing levels back up into the optimal range. PTH achieves this increase in remodeling primarily through its actions on the osteoblast. PTH increases the expression of the Notch ligand Jagged1 (JAG1) in osteoblasts [Weber et al., 2006].

1.4.1.4 Regulation

Osteoblasts can regulate the expansion of the hematopoietic stem cell niche in bone marrow through a Notch-mediated mechanism, and by increasing JAG1 expression on osteoblasts. PTH leads to an expansion of the hematopoietic lineage from which the osteoclasts are derived. Osteoblasts respond to PTH, as well as interleukin-11 (IL-11), prostaglandin E2 (PGE2), and 1,25(OH)\(_2\)D, by increasing the expression of RANKL and other osteoclast regulatory cytokines leading to increased osteoclast differentiation and activity and decreased osteoclast apoptosis [Atkins et al., 2003]. PTH induces increased neutral protease expression by osteoblasts and causes osteoblasts to contract away from the bone surface, exposing the bone and providing the osteoclasts access to the surface. Systemic release of PTH can induce increased bone resorption and \( \text{Ca}^{2+} \) release by enhancing osteoclast formation and activity, by increasing osteoblast-mediated preparation of the bone surface by neutral protease
secretion, and by providing the osteoclasts access to the bone surface by causing contraction of lining osteoblasts away from the bone.

1.4.1.5 ROS modulation of signaling pathways in bone cells.

(a) ROS promote bone loss by inhibiting osteoblast differentiation and enhancing osteoclastogenesis.

(b) ROS induced bone resorption occurs directly or indirectly (increased RANKL expression) through the modulation of kinases and transcription factor activities in both osteoclasts and osteoblasts [Wauquier et al., 2009].

Figure XII. ROS modulation of signaling pathways in bone cells [Wauquier et al., 2009].

1.4.2 Bone Remodeling

Bone remodeling is a surface phenomenon. It occurs on periosteal, endosteal, Haversian canal, and trabecular surfaces. Bone surfaces may be undergoing formation or resorption, or they may be inactive. These processes occur throughout life in both cortical and trabecular bone. In normal bone, matrix remodeling of bone is constant; up to 10% of all bone mass may be undergoing remodeling at any point in time. This process is called bone remodeling [Raisz, 2005].
1.4.2.1 Basic Multicellular Units or Bone Metabolic Units (BMUs)

Bone remodeling is accomplished by assembly of osteoclasts and osteoblasts into discrete temporary anatomic structural units called basic multicellular units or bone metabolic units (BMUs) as first described by Frost in 1963 [Frost and Thomas, 1963; Kaplan et al., 1994; Jilka, 2003].

Properties

(a) At the level of BMU, bone resorption and bone formation are tightly coupled in time and space.

(b) Bone mass is determined by two important factors, first by the balance between resorption and formation within a BMU and second by the number of BMUs active during a certain period of time in the given part of bone.

(c) The lifespan of osteoclasts and osteoblasts is short compared to the lifespan of the BMU; therefore, they must be continually replenished for BMU progression to occur.

(d) Bone loss and osteoporosis are determined by the imbalance between bone formation and resorption at the BMU and by the increased number of BMUs.

(e) The quantity of bone lost at the level of one BMU is very small, bone loss is driven mainly by the number of BMUs [Morgan et al., 2008].
1.4.3 Bone remodeling and oxidative stress.

(a) BMSCs, hematopoietic precursors differentiate into multinucleated osteoclasts in the presence of RANKL, expressed by osteoblasts.

(b) RANKL binding to the receptor RANK at the surface of pre-osteoclasts stimulates cell fusion.

(c) It activates resorption capabilities and enhances cell survival.

(d) OPG is a decoy receptor for RANKL and it prevents osteoclast differentiation.

(e) By degrading bone, osteoclasts create lacunae that are filled with newly synthesized matrices by osteoblasts.

(f) As a result of oxidative stress this bone coupling is unbalanced, and bone formation by osteoblasts is reduced, whereas osteoclast differentiation and activities and subsequent bone resorption are enhanced directly or indirectly through an increased RANKL production [Wauquier et al., 2009].

Figure XIV: Bone Remodeling and Oxidative stress [Wauquier et al., 2009].
1.5 Defining Osteoporosis (OP)

Osteoporosis (OP) is a chronic progressive metabolic bone-related disease that is characterized by low or decrease in bone mineral density (BMD) and microarchitectural deformation of bone tissue that leads to an increased risk of bone fragility and fractures and electrolyte imbalance [Kanis, 2002; Kim et al., 2013; Jin et al., 2014; Morgan et al., 2014]. Decrease in bone mass and deterioration of bone architecture are determined by bone turnover abnormalities. BMD is a medical term normally referring to the amount of mineral matter per square centimeter of bones. It is used in clinical medicine as an indirect indicator of osteoporosis. Osteoporosis has become an alarming health problem through the entire world and about 200 million people in the world are under the threat of this deleterious health problem [Lampropoulos et al., 2012; Roy, 2013]. With the extension of life expectancy, osteoporosis is becoming a major public health problem severely affecting the life quality of the elderly [Jin et al., 2014].

1.5.1 Definition

Osteoporosis is defined by the World Health Organization (WHO) as a BMD of 2.5 standard deviations or more below the mean peak bone mass (average of young, healthy adults) as measured by dual-energy X-ray absorptiometry (DEXA); the term "established osteoporosis" includes the presence of a fragility fracture [WHO, 1994].

1.5.2 Classification of Osteoporosis Disease

The disease may be classified as primary type 1, primary type 2, or secondary and that, their details are illustrated as under.

1.5.2.1 Primary Osteoporosis

Primary osteoporosis is mainly a disease of the elderly, the result of the cumulative impact of bone loss and deterioration of bone structure that occurs as people age [Seeman, 2003]. This form of osteoporosis is sometimes referred to as age-related osteoporosis.
1.5.2.1.1 Primary Type 1 Osteoporosis

The form of osteoporosis most common in women after menopause is referred to as primary type 1 or postmenopausal osteoporosis (PMO) [Fraser et al., 2011]. PMO is caused by an imbalance of bone metabolism induced by the promotion of bone resorption due to a lack of estrogen during menopause [Saika et al., 2001]. As much as 20% of bone mass can be lost in the first 5–7 years following menopause [Rayalam et al., 2011]. In postmenopausal osteoporosis estrogen deficiencies lead to high bone turnover and bone loss [Wensel et al., 2011].

1.5.2.1.2 Primary Type 2 Osteoporosis

Primary type 2 osteoporosis or senile osteoporosis occurs after age 75 and is seen in both females and males at a ratio of 2:1 [Fraser et al., 2011]. Women have two phases of age-related bone loss, which are as under:

(a) Rapid phase that begins at menopause and lasts 4–8 years. It results in losses of 5–10 percent of cortical bone and 20–30 percent of trabecular bone.

(b) Slower continuous phase that lasts throughout the rest of life [Riggs et al., 2002]. It results in losses of 20–25 percent of cortical and trabecular bone in both men and women, but over a longer period of time [Riggs et al., 2002].

As a result, women typically lose more bone than do men. Men go through only the slow, continuous phase. Around the ages of 30–35, cancellous or trabecular bone loss begins. Women may lose as much as 50%, while men lose about 30%.
Figure XV. Bone Loss in Postmenopausal Women and Aging Men, a schematic Representation. Source: Riggs et al., 1998.

1.5.2.2 Secondary Osteoporosis

Secondary osteoporosis may arise at any age and affect men and women equally. This form results from chronic predisposing medical problems or disease, or prolonged use of medications such as glucocorticoids, when the disease is called steroid-induced osteoporosis (SIOP) or glucocorticoid-induced osteoporosis (GIOP) [Khosla et al., 1994]. The majority of men with osteoporosis exhibit secondary causes of the disease [Orwoll, 1998]. The occurrence of OP is prevalent among the aging women than the aging men although corticosteroid treatment, intake of excessive alcohol, cigarette smoking, low calcium intake and hypogonadism may be the secondary cause [Lampropoulos et al., 2012; Chen et al., 2013; Roy, 2013].

1.5.3 Symptoms

OP is often described as a silent disease because it is typically asymptomatic until a fracture occurs, the disease negatively and significantly impacts morbidity and mortality as it can lead to severe pain, deformity, disability, and death [Chen et al.,
Introduction

Osteoporotic fractures occur in situations where healthy people would not normally break a bone; they are therefore regarded as fragility fractures. The signs of OP are deterioration of the microstructure of bone specifically at trabecular sites including vertebrae, ribs and hips, culmination in fragility fractures, pain and disability [Chen et al., 2013; Wongdee and Charoenphanhdu, 2011].

Figure XVI: Bone Fracture Areas in Osteoporosis. Source: NOF 2004.

1.5.4 Osteoporotic Fractures

Fractures are the most dangerous aspect of osteoporosis. Debilitating acute and chronic pain in the elderly is often attributed to fractures from osteoporosis and can lead to significant morbidity, deterioration of quality of life, and even mortality [Old and Calvert, 2004; Wariaghli et al., 2010; Goel and Kar, 2010; Zivna et al., 2013]. These fractures may also be asymptomatic. Multiple vertebral fractures lead to a
stooped posture, loss of height, and chronic pain with resultant reduction in mobility [Kim and Vaccaro, 2006]. Involvement of multiple vertebral bodies leads to kyphosis of the thoracic spine, leading to what is known as dowager’s hump. Fracture risk calculators assess the risk of fracture based upon several criteria, including BMD, age, smoking, alcohol usage, weight, and gender. It is known that the risk of fracture strongly correlates with BMD and the best site for the hip fracture risk prediction is the measurement of the proximal femur BMD [Wariaghli et al., 2010; Zivna et al., 2013]. Eighty percent of those diagnosed with osteoporosis are female, and in women the risk of hip fracture due to osteoporosis is equal to the combined risk of breast, uterine and ovarian cancer [U.S. Department of Health and Human Services, 2004]. In 2005, osteoporosis-related fractures were responsible for an estimated $19 billion in costs. By 2025, these costs are predicted to rise to approximately $25.3 billion, and over the next 50 years, the national cost may be as high as $240 billion [U.S. Department of Health and Human Services, 2004].

1.6 Pathogenesis of Disease

Bone structural integrity is maintained by removal of old bone by osteoclasts and synthesis of new bone in its place by osteoblasts [Jilka, 2003]. Bone is resorbed by osteoclast cells, after which new bone is deposited by osteoblast cells. The underlying mechanism in all cases of osteoporosis is an imbalance between bone resorption and bone formation.

1.6.1 Mechanism

There are the three following main mechanisms by which osteoporosis develop:

(a) An inadequate peak bone mass

(b) Excessive bone resorption

(c) Inadequate formation of new bone during remodeling.

It should be noted that an inadequate peak bone mass means that the skeleton develops insufficient mass and strength during growth. Interplay of these three mechanisms underlies the development of fragile bone tissue [Raisz, 2005].
1.6.2 Role of Estrogen

Hormonal factors strongly determine the rate of bone resorption. Lack of estrogen increases bone resorption, as well as decreasing the deposition of new bone that normally takes place in weight-bearing bones. It should be noted that lack of estrogen happens as a result of menopause. The amount of estrogen needed to suppress this process is lower than that normally needed to stimulate the uterus and breast gland. The $\alpha$-form of the estrogen receptor appears to be the most important in regulating bone turnover [Raisz, 2005].

1.6.3 Role of Calcium Metabolism, Vitamin D, Prostaglandin and Parathyroid Hormone

The activated vitamin D3 (1, 25(OH)2 D3), prostaglandin E2 and the parathyroid hormone act on osteoblasts, and control the differentiation of osteoclasts through the expression of RANKL [Takahashi et al., 1988a; Suda et al., 1999]. Calcium metabolism plays a significant role in bone turnover, and deficiency of calcium and vitamin D leads to impaired bone deposition. The parathyroid glands react to low calcium levels by secreting parathyroid hormone, which increases bone resorption to ensure sufficient calcium in the blood [Raisz, 2005].

1.6.4 OPG/RANKL/RANK system

The discovery of the molecular triad OPG/ RANK/ RANKL system in the mid 1990s for the regulation of bone resorption has led to major advances in our understanding of how bone modeling and remodeling are regulated [Boyce and Xing, 2008]. This system has helped in elucidating a key signaling pathway between stromal cells and osteoclasts [Wittrant et al., 2004]. This system is the dominant and final mediator of osteoclastogenesis. OPG, RANKL and RANK are the members of the tumor necrosis factor (TNF) super family of ligands and receptors. These three factors play a decisive role in regulating bone metabolism. This was demonstrated by the findings of extremes of skeletal phenotypes (osteoporosis vs. osteopetrosis) in mice with altered expression of these molecules [Khosla, 2001]. These TNF super family members also have important functions outside bone [Boyce and Xing, 2007].
1.6.4.1 Osteoprotegerin (OPG)

It is also known as osteoclastogenesis inhibitory factor (OCIF) or tumor necrosis factor receptor super family member 11B (TNFRSF11B). It is a protein that in humans is encoded by the TNFRSF11B gene [Wada et al., 2006]. The initial cloning and characterization of OPG as a soluble, decoy receptor for RANKL belonging to the TNF receptor super family was the first step that eventually led to an unraveling of this system [Simonet et al., 1997; Khosla, 2001].

![Figure XVII: The Signaling Pathway for normal osteoclastogenesis](Boyte and Xing, 2007).

**Properties**

(a) OPG is a basic glycoprotein comprising 401 amino acid residues arranged into 7 structural domains.
(b) OPG is found as either a 60-kDa monomer or 120-kDa dimer linked by disulfide bonds [Schoppet et al., 2002].

(c) RANKL activity can be blocked by the soluble decoy receptor OPG, resulting in prevention of bone resorption [Simonet et al., 1997].

(d) OPG, a recently described member of the TNF receptor super family, is produced by a lot of cell types, such as bone-marrow stromal cells and osteoblasts.

(e) OPG protects bone from excessive resorption by binding to RANKL and preventing it from binding to RANK [Boyce and Xing, 2008].

(f) OPG blocks the fusion/differentiation stage of osteoclast precursors, rather than the proliferation stage, by binding to RANKL.

(g) Thus, the relative concentration of RANKL and OPG in bone is a major determinant of bone mass and strength [Boyce and Xing, 2008].

(h) OPG can reduce the production of osteoclasts by inhibiting the differentiation of osteoclast precursors into osteoclasts and also regulates the resorption of osteoclasts in vitro and in vivo.

(i) Thus, in this way, OPG protects the skeleton from excessive bone resorption by binding to RANKL and preventing it from binding to its receptor, RANK [Raisz, 2005; Boyce and Xing, 2007].

(j) By binding RANKL, OPG inhibits NF-κB which is a central and rapid acting transcription factor for immune-related genes, and a key regulator of inflammation, innate immunity, and cell survival and differentiation [Krakauer, 2008].

(k) OPG levels are influenced by voltage-dependent calcium channels Cav1.2.

(l) OPG also protects arteries from medial calcification [Boyce and Xing, 2007].
Space shuttle flight STS-108 in 2001 tested the effects of OPG on mice in microgravity, finding that it did prevent increase in resorption and maintained mineralization [Bateman and Countryman, 2002].

OPG production is stimulated in vivo by the female sex hormone estrogen as well as the osteoporosis drug, strontium ranelate [Khosla, 2001].

1.6.4.2 Receptor activator of NF-κB ligand (RANKL)

RANKL is the molecule which is blocked by OPG. It is also known as tumor necrosis factor ligand super family member 11 (TNFSF11), TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL) and osteoclast differentiation factor (ODF) is a protein that in humans is encoded by the TNFSF11 gene [Wong et al., 1997; Anderson et al., 1997; Yasuda et al., 1998; Lacey et al., 1998].

Properties

(a) RANKL is one of the critical mediators of osteoclastogenesis [Bharti et al., 2004].

(b) RANKL is the most important locally produced pro-osteoclastogenic factor/cytokine that, in combination with M-CSF, induces osteoclast formation in vitro [Wittrant et al., 2004].

(c) RANKL is expressed as a membrane-bound protein on the surface of osteoblasts, osteocytes and marrow stromal cells [Lacey et al., 1998].

(d) In addition, activated T cells secrete RANKL as a soluble molecule [Kong et al., 1999].

(e) Most osteotropic factors such as IL-1, IL-11, PGE2 and 1,25-(OH)$_2$D$_3$ induce osteoclast formation by binding to marrow stromal cells, which in turn express increased levels of soluble or membrane forms of RANKL [Wittrant et al., 2004].

(f) It was identified as the key mediator of osteoclastogenesis in both a membrane-bound form expressed on preosteoblastic/stromal cells and T cells
as well as a soluble form [Suda et al., 1999; Wong et al., 1999; Khosla, 2001; Theill et al., 2002].

(g) Under physiologic conditions, osteoblasts produce CSF-1 and the differentiation-inducing factor, RANKL [Yoshida et al., 1990; Amano et al., 1998; Lacey et al., 1998; Yasuda et al., 1998].

(h) Osteoclastic activity is triggered via the osteoblasts' surface-bound RANKL activating the osteoclasts' surface-bound RANK.

(i) The binding of RANKL to the RANK receptor activates NF-κB signaling leading to the formation of mature multinucleated osteoclasts [Wada et al., 2006].

(j) The activity of RANKL is balanced by the level of expression of its inhibitor osteoprotegerin OPG.

(k) It is the local ratio of RANKL to OPG that ultimately determines if osteoclast formation will occur by regulating the amount of available RANKL.

(l) Therefore, RANKL/OPG ratio is an important determinant of bone mass and skeletal integrity [Boyce and Xing, 2007].

(m) RANKL induces osteoclastogenesis through the activation of NF-κB [Bharti et al., 2004].

1.6.4.3 Receptor Activator of Nuclear Factor κ B (RANK)

RANK (receptor activator of nuclear factor κB) is stimulated by RANKL. RANK is also called osteoclast differentiation factor receptor (ODFR), TNF receptor super family member 11A (TNFRSF11A) and TNF-related activation induced cytokine receptor (TRANCE-R) [Wada et al., 2006].

Properties

(a) RANK is also expressed on dendritic cells and facilitates immune signaling.
RANKL then binds to its receptor RANK, present at the surface of osteoclast precursors and mature osteoclasts, inducing osteoclast formation and activation [Nakagawa et al., 1998; Khosla, 2001].

The interaction between RANK and RANKL plays a critical role in promoting osteoclast differentiation and activation leading to bone resorption.

The binding of RANKL to its receptor RANK leads to recruitment of the adaptor protein TNF receptor-associated factor 6 (TRAF6) to the cytoplasmic domain of RANK, thereby resulting in the activation of distinct signaling cascades mediated by MAPK, including JNK, p38 MAP kinase (p38), and extracellular signal-regulated kinase (ERK), leading NF-κB activation [Boyle et al., 2003].

NF-κB increases c-Fos expression and c-Fos interacts with NFATc1 to trigger the transcription of osteoclastogenic genes leading to the osteoclast differentiation [Jilka, 2003; Raisz, 2005; Boyce and Xing, 2007].

JNK1- activated c-Jun signaling in cooperation with NFAT is a key to RANKL-regulated osteoclast differentiation [Ikeda et al., 2004].

Stimulation of p38 results in the downstream activation of the microphthalmia/microphthalmia transcription factor (mi/Mitf).

This factor controls the expression of the genes encoding TRAP and CTSK, indicating the importance of p38 signaling cascades [Boyle et al., 2003].

RANKL-induced NFATc1 is a downstream event of NF-κB signal pathway [Moon et al., 2012].

RANKL/RANK signaling regulates osteoclast formation, activation and survival in normal bone modeling and remodeling and in a variety of pathologic conditions characterized by increased bone turnover.

RANKL/RANK signaling is also required for lymph node formation and mammary gland lactational hyperplasia [Boyce and Xing, 2007].
1.6.5 Regulation of Bone Turnover

Bone turnover is characterized by the formation of new bone by osteoblasts and the resorption of old bone by osteoclasts. Local production of eicosanoids and ILs is thought to participate in the regulation of bone turnover, and excess or reduced production of these mediators may underlie the development of osteoporosis [Raisz, 2005].

1.7 Diagnosis

The diagnosis of osteoporosis can be made using conventional radiography and by measuring BMD [Guglielmi and Scalzo, 2010]. The diagnosis of osteoporosis also requires investigations into potentially modifiable underlying causes; this may be done with blood tests.

1.7.1 Conventional Radiography

Conventional radiography is useful for detecting complications of osteopenia, such as fractures; for differential diagnosis of osteopenia; or for follow-up examinations in specific clinical settings, such as soft tissue calcifications, secondary hyperparathyroidism, or osteomalacia in renal osteodystrophy. Osteopenia is basically the reduced bone mass; it is also called as preosteoporosis.

1.7.2 Dual-Energy X-ray Absorptiometry (DEXA)

DEXA is the most popular method of measuring BMD. DEXA is considered the gold standard for the diagnosis of osteoporosis. Osteoporosis is diagnosed when the BMD is less than or equal to 2.5 standard deviations below that of a young (30–40-year-old, healthy adult women reference population [WHO, 2003]. This is translated as a T-score. But because bone density decreases with age, more people
become osteoporotic with increasing age [WHO, 2003]. WHO has established the following diagnostic guidelines [WHO, 1994; WHO, 2003].

**Table 2: Relationship between T-Score Ranges and Disease State.**

<table>
<thead>
<tr>
<th>Category</th>
<th>T-Score Range</th>
<th>% Young Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>T-Score$\geq$-1.0</td>
<td>85%</td>
</tr>
<tr>
<td>Osteopenia</td>
<td>-2.5$&lt;\text{T-Score}&lt;-1.0$</td>
<td>14%</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>T-Score$\leq$-2.5</td>
<td>0.6%</td>
</tr>
<tr>
<td>Severe Osteoporosis</td>
<td>T-Score$\leq$-2.5 with fragility fracture</td>
<td></td>
</tr>
</tbody>
</table>

**1.8 Bone Turnover Markers (BTMs)**

Biochemical bone turnover markers (BTMs) can be divided into two groups:

(a) Markers of bone formation

(b) Markers of bone resorption.

However, in disease states in which both processes are coupled and disclose similar increase, BTMs reflect the overall rate of bone turnover. In such cases, levels of the bone resorption markers are correlated positively with histomorphometric parameters of bone formation. BTMs cannot discriminate between bone turnover changes in the cortical and trabecular envelopes. The increase in the BTM levels is followed by an increase in BMD during puberty but by a decrease in BMD after menopause.

**Table 3: Biochemical Markers for Bone Remodeling**

<table>
<thead>
<tr>
<th>Markers of bone formation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum</strong></td>
</tr>
<tr>
<td>Osteocalcin (bone Gla protein) [Li et al., 2011]</td>
</tr>
<tr>
<td>*Total and bone ALP [Li et al., 2011]</td>
</tr>
<tr>
<td>*N-terminal propeptide of type I collagen (P1NP)</td>
</tr>
</tbody>
</table>
**C-terminal propeptide of type I collagen (PICP)**

### Markers of bone resorption

#### Plasma/serum

- Tartrate-resistant acid phosphatase (TRACP)
- Pyridinoline (PYD) and deoxypyridinoline (DPD)
- C-terminal cross-linking telopeptide of type I generated by metalloproteinases (CTX-MMP)
- *C-terminal cross-linking telopeptide of type I collagen (CTX-I)*
- N-terminal cross-linking telopeptide of type I collagen (NTX-I)

#### Urine

- Pyridinoline (PYD) and deoxypyridinoline (DPD)
- C-terminal cross-linking telopeptide of type I collagen (CTX-I)
- N-terminal cross-linking telopeptide of type I collagen (NTX-I)
- Calcium
- Hydroxyproline
- Galactosyl-hydroxylysine

*ALP acts as a transmembrane receptor involved in osteoprogenitor–osteoblast adhesion, migration and differentiation [Hui et al., 1993; Li et al., 2011].

*PINP is marker of collagen metabolism before its integration to bone matrix and reflects a bone formation [Zivna et al., 2013].

*CTX-I is released from C-terminal part of telopeptide of collagen I by proteolytic enzymes and its detection is a sensitive indicator of bone resorption [Herrmann et al., 2008; Zivna et al., 2013].

### 1.9 Reactive Oxygen Species (ROS)

ROS are chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides. ROS form as a natural byproduct of the normal metabolism.
of oxygen and have important roles in cell signaling and homeostasis [Devasagayam et al., 2004]. However, during times of environmental stress, ROS levels can increase dramatically [Devasagayam et al., 2004]. This may result in significant damage to cell structures. Cumulatively, this is known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation.

**Figure XVIII: Electron structures of common reactive oxygen species, Source: [http://www.biotek.com/resources/articles/reactive-oxygen-species.html](http://www.biotek.com/resources/articles/reactive-oxygen-species.html)**

### 1.9.1 Oxidative Stress

Oxidative stress is caused by an imbalance between the systemic manifestation of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage or antioxidant defense [Morgan et al., 2014]. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and deoxyribonucleic acid (DNA). Some ROS act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. Cellular protective mechanisms against oxidative stress include transcriptional control of cytoprotective enzymes by the transcription factor, nuclear factor E2-related factor 2 (Nrf2) [Kanzaki et al., 2014]. Recently, it has been reported that loss of ovarian function causes oxidative stress as well as bone loss. ROS induced by the failure of ovarian function are responsible for the bone loss by increasing the number of osteoclasts (OC). ROS enhanced OC
survival via Src homology 2 domain-containing phosphatase-1 (SHP-1), c-Src, Akt, and ERK. Thus, the association and oxidation of c-Src and SHP-1 by ROS are key steps in enhancing OC survival, which are responsible for increased bone loss when ovarian function ceases [Ke et al., 2014].

1.9.2 Oxidative Damage

In aerobic organisms the energy needed to fuel biological functions is produced in the mitochondria via the electron transport chain (ETC). ROS with the potential to cause cellular damage are also produced in addition to energy. ROS can damage DNA, RNA and proteins, which, contributes to the physiology of ageing. Hydrogen peroxide (H$_2$O$_2$) is a major contributor to oxidative damage. It is converted from superoxide that leaks from the mitochondria. Catalase and superoxide dismutase ameliorate the damaging effects of H$_2$O$_2$ and superoxide, respectively, by converting these compounds into oxygen and water. This conversion is not 100% efficient, and residual peroxides persist in the cell. While ROS are produced as a product of normal cellular functioning, excessive amounts can cause deleterious effects [Patel et al., 1999].

1.9.3 ROS Scavengers

1.9.3.1 Enzymes

Normally, cells defend themselves against ROS damage with enzymes such as

(a) Alpha-1-1microglobulin
(b) Superoxide dismutases (SOD)
(c) Catalases
(d) Lactoperoxidases
(e) Glutathione peroxidases (GPx)
(f) Peroxiredoxins
(g) Thioredoxins

1.9.3.2 Cellular Antioxidants
Some small molecular antioxidants also play important roles as cellular antioxidants such as

(a) Ascorbic acid (vitamin C)
(b) Tocopherol (vitamin E)
(c) Uric acid
(d) Glutathione

1.9.3.3 Polyphenol Antioxidants

Polyphenol antioxidants assist in preventing ROS damage by scavenging free radicals.

1.9.3.4 Plasma antioxidant

The antioxidant ability of the extracellular space is less - e.g., the most important plasma antioxidant in humans is uric acid.

1.9.4 Useful Effects of ROS

Effects of ROS on cell metabolism are well documented in a variety of species.

(a) Roles in apoptosis or programmed cell death

(b) The induction of host defense genes [Rada and Leto, 2008; Conner et al., 2002].

(c) Mobilization of ion transport systems

(d) Control of cellular function such as platelets involved in wound repair and blood homeostasis release ROS to recruit additional platelets to sites of injury.

(e) These also provide a link to the adaptive immune system via the recruitment of leukocytes.
1.9.5 Harmful Effects of ROS

According to [Brooker, 2011], the harmful effects of ROS on the cell are most often:

(a) damage of DNA
(b) oxidations of polyunsaturated fatty acids in lipids called lipid peroxidation
(c) oxidations of amino acids in proteins
(d) oxidatively inactivate specific enzymes by oxidation of co-factors

1.9.6 Cellular localization of ROS production.

Figure XIX: Cellular localization of ROS production [Wauquier et al., 2009].

(a) ROS are produced in cells at various sites including the plasma membrane, mitochondria and cytoplasm.
(b) The largest amounts of ROS are produced in mitochondria because of electron leaks in the respiratory chain.
At the surface of the cell, NADPH oxidases transport electrons across the plasma membrane to generate superoxide (O$_2^-$).

Superoxide is unstable and so is rapidly converted to H$_2$O$_2$.

H$_2$O$_2$ can diffuse through the cell membrane [Wauquier et al., 2009].

1.9.6 Types of ROS

1.9.6.1 Exogenous ROS

Exogenous ROS can be produced from pollutants, tobacco, smoke, drugs, xenobiotics, or radiation.

1.9.6.1.1 Radiolysis

Ionizing radiation can generate damaging intermediates through the interaction with water, a process termed radiolysis. Since water comprises 55-60% of the human body, the probability of radiolysis is quite high under the presence of ionizing radiation.

1.9.6.1.1.1 Mechanism

In the process, water loses an electron and become highly reactive. Then through a three-step chain reaction, water is sequentially converted to hydroxyl radical (OH), H$_2$O$_2$, superoxide radical (·O$_2^-$) and ultimately oxygen (O$_2$). The hydroxyl radical is extremely reactive that immediately removes electrons from any molecule in its path, turning that molecule into a free radical and so propagating a chain reaction. But H$_2$O$_2$ is actually more damaging to DNA than hydroxyl radical since the lower reactivity of H$_2$O$_2$ provides enough time for the molecule to travel into the nucleus of the cell, subsequently wreaking havoc on macromolecules such as DNA.

1.9.6.2 Endogenous ROS

There are multiple sources of ROS in the cell including NADPH oxidase (NOX) [Block and Gorin, 2012], xanthine oxidase (XO), uncoupling of nitric oxide synthase (NOS), cytochrome P450, and mitochondrial ETC [Li et al., 2013]. ROS are produced intracellularly through multiple mechanisms and depending on the cell and tissue types.
**1.9.6.2.1 Role of Mitochondria**

Mitochondria convert energy for the cell into a usable form, adenosine triphosphate (ATP) [Li et al., 2013]. The process, in which ATP is produced, is called oxidative phosphorylation which involves the transport of protons across the inner mitochondrial membrane by means of the ETC. In ETC, electrons are passed through a series of proteins via oxidation-reduction reactions, with each acceptor protein along the chain having a greater reduction potential than the previous. An oxygen molecule is the last destination for an electron along this chain.

**1.9.6.2.2 Superoxide radical Generation**

In normal conditions, the oxygen is reduced to produce water. In about 0.1–2% of electrons passing through the chain, oxygen is instead prematurely and incompletely reduced to give the superoxide radical ($\cdot O_2^-$). [Li et al., 2013]. Superoxide is not particularly reactive by itself, but can inactivate specific enzymes or initiate lipid peroxidation in its protonated form, hydroperoxyl $HO_2\cdot$. The pKa of hydroperoxyl is 4.8. Thus, at physiological pH, the majority will exist as superoxide. Mitochondrial ROS (mt ROS) directly stimulate the production of proinflammatory cytokines [West et al., 2011] and pathological conditions as diverse as malignancies, autoimmune diseases, and cardiovascular diseases all share common phenotype of increased mtROS production above basal levels [Li et al., 2013]. Recently it has been reported that mitochondrial-derived reactive oxygen species (mt ROS) regulate the strength of postsynaptic GABA$_A$ receptors at inhibitory synapses of cerebellar stellate cells. GABA, i.e., $\gamma$-aminobutyric acid is the chief inhibitory neurotransmitter in the mammalian central nervous system [Accardi et al., 2014]

**1.9.6.2.3 ROS and Apoptosis**

If too much damage is present in mitochondria, a cell undergoes apoptosis or programmed cell death.

**1.9.6.2.3.1 Mechanism**

(1) B-cell lymphoma 2 (Bcl-2) proteins are layered on the surface of the mitochondria, detect damage, and activate a class of proteins called bcl-2-associated X proteins (BAX).
Bax punch holes in the mitochondrial membrane, causing cytochrome C to leak out.

This cytochrome C binds to Apaf-1, or apoptotic protease activating factor-1, which is free-floating in the cell's cytoplasm.

The Apaf-1 and cytochrome C bind together to form apoptosomes by using energy from the ATPs in the mitochondrion.

The apoptosomes bind to and activate caspase-9, another free-floating protein.

The caspase-9 then cleaves the proteins of the mitochondrial membrane, causing it to break down and start a chain reaction of protein denaturation and eventually phagocytosis of the cell.

1.9.7 Antioxidant Enzymes

There are three major types of primary intracellular antioxidant enzymes in mammalian cells:

(a) Superoxide dismutase (SOD)
(b) Catalase
(c) Peroxidase, of which glutathione peroxidase (GPx) is the most prominent.

Figure XX: Antioxidant enzyme schematic [Weydert and Cullen, 2010].
1.9.8 Superoxide Dismutase (SOD)

Superoxide Dismutases (SOD) are the enzymes that catalyze the dismutation of superoxide (\(-\text{O}_2^−\)) into oxygen and \(\text{H}_2\text{O}_2\) [Li et al., 2013]. They convert \(\text{O}_2^−\) into \(\text{H}_2\text{O}_2\) [Weydert and Cullen, 2010]. Thus, they are an important antioxidant defence in nearly all cells exposed to oxygen.

1.9.7.1 Reaction Catalyzed

The SOD-catalysed dismutation of superoxide may be written with the following half-reactions:

\[
\begin{align*}
\text{M}^{(n+1)+} - \text{SOD} + \text{O}_2^- & \rightarrow \text{M}^{n+} - \text{SOD} + \text{O}_2 \\
\text{M}^{n+} - \text{SOD} + \text{O}_2^- + 2\text{H}^+ & \rightarrow \text{M}^{(n+1)+} - \text{SOD} + \text{H}_2\text{O}_2.
\end{align*}
\]

Where M = Cu (n=1); Mn (n=2); Fe (n=2); Ni (n=2).

In this reaction the oxidation state of the metal cation oscillates between n and n+1.

1.9.7.2 SODs in Higher Plants

SOD isozymes have been localized in different cell compartments.

1.9.7.2.1 Mn-SOD

Mn-SOD is present in mitochondria and peroxisomes.

1.9.7.2.2 Fe-SOD

Fe-SOD has been found mainly in chloroplasts but has also been detected in peroxisomes.

1.9.7.2.3 CuZn-SOD

CuZn-SOD has been localized in cytosol, chloroplasts, peroxisomes, and apoplast [Corpas et al., 2001; Corpas et al., 2006].

1.9.7.3 SODs in Humans

Three isoforms of SOD have been identified in humans, in all other mammals, and most chordates [Li et al., 2013].
(a) SOD1/copper-zinc SOD (CuZn-SOD),
(b) SOD2/manganese SOD (Mn-SOD), and
(c) Extracellular SOD3 (EC-SOD).

Three forms of superoxide dismutase are present

1.9.7.3.1 SOD1

SOD1 is widely distributed throughout the cell cytoplasm, nucleus, and intermembrane space of mitochondria [Okado-Matsumoto and Fridovich, 2001]. SOD1 is a dimer (consists of two units). SOD1 contains copper and zinc. The genes are located on chromosome 21 (21q22.1).

1.9.7.3.2 SOD2

SOD2 is expressed only in the mitochondrial matrix [Okado-Matsumoto and Fridovich, 2001]. SOD2 is a tetramer (consists of four subunits). SOD2 has manganese in its reactive centre. The genes are located on chromosome 6 (6q25.3). The deficiency of SOD2 causes early neonatal death in gene knockout mice [Li et al., 1995] and endothelial dysfunction in carotid artery of proatherogenic apolipoprotein E (ApoE)-deficient mice [Madamanchi and Runge, 2007; Ohashi et al., 2006].

1.9.7.3.3 SOD3

SOD3 is found in the extracellular space [Li et al., 2013]. SOD3 is a dimer (consists of four subunits). SOD3 contains copper and zinc. The genes are located on chromosome 4 (4p15.3-p15.1).

1.9.8 Catalase

The catalases (CAT) and peroxidases (GPx) convert H$_2$O$_2$ into water [Weydert and Cullen, 2010]. CAT is a heme-containing tetramer of four polypeptide chains that reduces H$_2$O$_2$ to water [Li et al., 2013]. Although catalase is highly efficient at reducing H$_2$O$_2$, it may not play a central role in scavenging ROS in the mitochondria since it is localized mainly in peroxisomes except that rat heart mitochondria does
partially depend on catalase to scavenge ROS [Radi et al., 1991]. If H$_2$O$_2$ removal is inhibited, then there is direct toxicity resulting from H$_2$O$_2$-mediated damage [Weydert and Cullen, 2010]. If CAT is inhibited, cells also cannot remove H$_2$O$_2$ [Weydert and Cullen, 2010].

\[
2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2
\]

1.9.9 Glutathione Peroxidase (GPx)

GPx is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage.

1.9.9.1 Biochemical Function

GPx requires several secondary enzymes, including glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD), and cofactors, including glutathione (GSH), NADPH and glucose 6-phosphate, to function at high efficiency [Weydert and Cullen, 2010]. The biochemical function of GPx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free H$_2$O$_2$ to water. GPx catalyzes the reductive inactivation of H$_2$O$_2$ by transferring the energy of the reactive peroxides to a very small sulphur-containing protein called glutathione using reduced glutathione (GSH) as a cofactor [Li et al., 2013]. GSH is a tripeptide containing of three amino acid residues including glutamate, cysteine, and glycine.

1.9.9.2 Reaction Catalyzed

The main reaction that GPx catalyzes is

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GS–SG} + 2\text{H}_2\text{O}
\]

During the process of reducing H$_2$O$_2$, GSH is oxidized to oxidized glutathione (GSSG). Where,

(a) GSH represents reduced monomeric glutathione

(b) GS–SG represents glutathione disulfide.
1.9.9.2.1 Mechanism of Reaction

(1) The mechanism involves oxidation of the selenol of a selenocysteine residue by H₂O₂.

(2) This process gives the derivative with a seleninic acid (RSeOH) group.

(3) The seleninic acid is then converted back to the selenol by a two step process that begins with reaction with GSH to form the GS-SeR and water.

(4) A second GSH molecule reduces the GS-SeR intermediate back to the selenol, releasing GS-SG as the by-product.

(5) A simplified representation is shown below [Bhabak and Mugesh, 2010].

- $\text{RSeH} + \text{H}_2\text{O}_2 \rightarrow \text{RSeOH} + \text{H}_2\text{O}$
- $\text{RSeOH} + \text{GSH} \rightarrow \text{GS-SeR} + \text{H}_2\text{O}$
- $\text{GS-SeR} + \text{GSH} \rightarrow \text{GS-SG} + \text{RSeH}$

1.9.9.3 Glutathione Reductase (GR)

GR then reduces the oxidized glutathione to complete the cycle:

- $\text{GS–SG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+$

Thus, GSSG is then recycled back to GSH by the enzyme GR using NADPH as a substrate [Handy and Loscalzo, 2012]. Thus, the maintenance of GSH for optimal scavenging capacity is dependent on the bioavailability of NADPH stores [Li et al., 2013]. If GR is inhibited, cells cannot remove H₂O₂ through the glutathione peroxidase system and the levels of glutathione disulfide (GSSG) increase [Weydert and Cullen, 2010].

Note: If glutathione synthesis is inhibited, either by inhibiting glutathione synthetase (GS) or by $\gamma$-glutamyl cysteine synthetase ($\gamma$-GCS), glutathione will be depleted and GPxs will not be able to remove H₂O₂ [Weydert and Cullen, 2010]. If glucose uptake is inhibited creating a chemically induced state of glucose deprivation, hydroperoxide detoxification will also be inhibited [Weydert and Cullen, 2010].
1.9.9.4 Selenium Reactive Centre

The selenium contained in these enzymes acts as the reactive centre, carrying reactive electrons from the peroxide to the glutathione. As the integrity of the cellular and subcellular membranes depend heavily on GPx, its antioxidative protective system itself depends heavily on the presence of selenium.

1.9.9.5 Isoenzyme of Glutathione Peroxidase

Several isozymes are encoded by different genes, which vary in cellular location and substrate specificity. So far, eight different isoforms of GPx (GPx1-8) have been identified in humans.

1.9.9.5.1 Glutathione Peroxidase 1 (GPx1)

GPx1 is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is H₂O₂. Mammalian GPx1 has been shown to be selenium-containing enzyme. GPx1 is a homotetrameric protein.

1.9.9.5.2 Glutathione Peroxidase 2 (GPx2)

GPx2 is an intestinal and extracellular enzyme. Mammalian GPx2 has been shown to be selenium-containing enzyme. GPx2 is a homotetrameric protein.

1.9.9.5.3 Glutathione Peroxidase 3 (GPx3)

GPx3 is extracellular, especially abundant in plasma [Muller et al., 2007]. Mammalian GPx3 has been shown to be selenium-containing enzyme. GPx3 is a homotetrameric protein.

1.9.9.5.4 Glutathione Peroxidase 4 (GPx4)

GPx4 has a high preference for lipid hydroperoxides. It is expressed in nearly every mammalian cell, though at much lower levels. Mammalian GPx4 has been shown to be selenium-containing enzyme. GPx4 has a monomeric structure.
1.9.5.5 Glutathione Peroxidase 6 (GPx6)

GPx6 is a selenoprotein in humans with cysteine-containing homologues in rodents.

1.9.10 Peroxiredoxins

Peroxiredoxins are a family of antioxidant enzymes that regulate cytokine-induced peroxide levels and mediate signal pathways [Rhee et al., 2005]. Peroxiredoxins also degrade H$_2$O$_2$, within the mitochondria, cytosol, and nucleus. There are six peroxiredoxins in this family. H$_2$O$_2$ has the highest affinity to peroxiredoxin 2 (100%), then to GSH (<0.01%), to Cdc25B (<0.0001%) and to protein tyrosine phosphatase 1B (<0.000001%), demonstrating the importance of peroxiredoxins [Winterbourn, 2008].

1.9.11 Thioredoxins

Thioredoxins are small proteins that play a variety of roles depending upon binding interactions and oxidoreductase activity. Mammalian thioredoxin-2 (Trx2) is a mitochondrial protein. Trx2 deficiency results in embryonic lethal at gestational day 10.5 and embryos show massive apoptosis. The timing coincides with the maturation of mitochondrial function [Li et al., 2013].

1.10 Tea (Camellia sinensis)

Tea is the most consumed beverage worldwide next to water. Tea is a flavonoid-rich beverage and contributes substantially to the intake of dietary catechins. Tea also contains some flavonols, particularly quercetin and kaempferol [McKay and Blumberg, 2007].

1.10.1 Physiological Effects of the Tea Polyphenol

Various physiological effects of the tea Polyphenol catechin for improving diseases such as cancer, arteriosclerosis, hyperlipemia, and osteoporosis have been reported [McKay and Blumberg, 2002; Crespy and Williamson, 2004; Kamon et al., 2010]. Flavonoids are commonly defined as dietary antioxidants and catechins can quench reactive oxygen and nitrogen species [Higdon and Frei, 2003], their bioactivity may
result as well from other mechanisms of action [Lotito and Frei, 2006], e.g., inhibition of inflammation [Wheeler et al., 2004], regulation of nitric oxide [Vita, 2003], stimulation of specific signal transduction pathways, and modulation of other cellular processes such as apoptosis [Lambert and Yang, 2003]. EGCG has various physiological functions such as the protection of DNA damage/methylation, suppression of protease activity, induction of apoptosis, and regulation of cell cycle and arrest, in the cell [Khan et al., 2006; Chen et al., 2008]. EGCG acts on a specific signaling pathway through binding to the 67 kDa laminin receptor (67LR) [Tachibana et al., 2004]. Tea polyphenol is known to mitigate osteoporosis as one of its physiological roles [Hegarty et al., 2000].

1.10.2 The Non Herbal Teas

All non-herbal teas, including green, oolong, black, and white teas, are derived from the leaves of the tropical evergreen *Camellia sinensis* [McKay and Blumberg, 2007].

1.10.2.1 Green Tea

About 20% of the consumed tea is green tea, which is primarily consumed in China and Japan and contains mostly nonoxidized Polyphenols, more particularly catechins [Graham, 1992; Mukhtar and Ahmad, 2000]. Green tea extract contains 85% polyphenols by weight. Composition of polyphenols in green tea extract used is shown as % total polyphenols (Zhong et al., 2003) (Fig. XXI). After harvesting, the leaves of the bush are steamed and dried to produce green tea leaves. In green tea leaves, the following polyphenolic compounds are found:

(a) (-)-epigallocatechin-3-gallate (EGCG): major component.
(b) (-)-epicatechin (EC),
(c) (-)-epigallocatechin (EGC) and
(d) (-)-epicatechin-3-gallate (ECG)

Note: (b), (c) and (d) are present in green tea at lower levels [Graham, 1992; Mukhtar and Ahmad, 2000]. ECG has been reported to stimulate osteoblast differentiation through a transcriptional activation [Byun et al., 2014].
Figure XXI: Molecular structures of *C. sinensis* polyphenols (Zhong et al., 2003).
Introduction

1.10.2.1.1 Epigallocatechin gallate (EGCG)

Epigallocatechin-3-gallate (EGCG) is the ester of epigallocatechin and gallic acid, and is a type of catechin. EGCG is the most abundant catechin in tea and is a potent antioxidant that may have therapeutic applications in the treatment of many disorders. EGCG accounts for more than 50% of the total of catechins [Nagal et al., 2006]. EGCG, a green tea polyphenol has been reported to exert potent anti-oxidant and anti-inflammatory effects by inhibiting signaling and gene expression [Yang et al., 2014].

1.10.2.1.2 EGCG and Osteoporosis

EGCG has the physiological role in the induction of osteoclast cell death [Nakagawa et al., 2002; Yun et al., 2007] and mineralization of osteoblasts [Takita et al., 2002; Vali et al., 2007]. EGCG is found to reduce the generation of TRAP positive multinucleated cells, bone resorption activity, and osteoclast-specific gene expression without affecting cell viability [Morinobu et al., 2008]. EGCG down-regulated expression of nuclear factor of activated T cells c1 (NF-ATc1), but not of NF-κB, c-
Fos, and c-Jun, suggesting that down regulation of NF-ATc1 is one of the molecular bases of EGCG action [Morinobu et al., 2008]. EGCG is found to suppress osteoclast differentiation and ameliorated experimental arthritis in mice over the short term [Morinobu et al., 2008]. The inhibitory effect of EGCG to osteoclastogenesis has been reported to be associated with a down regulation of RANKL/RANK signal, and increased apoptosis of preosteoclasts [Zhao et al., 2014]. Mah et al has reported EGCG at a low concentration can slightly enhance the osteogenic effect in vivo, whereas at a higher concentration it can prevent the osteogenic differentiation of human alveolar bone-derived cells (hABCs) both in vitro and in vivo [Mah et al., 2014].

1.10.2.1.3 Effect of Higher Temperature

In a high temperature environment, an epimerization change is more likely to occur. As exposure to boiling water for 30 straight minutes leads to only a 12.4% reduction in the total amount of EGCG, the amount lost in a brief exposure is insignificant. Even when special conditions were used to create temperatures well above that of boiling water, the amount lost increased only slightly [Wang et al., 2008]. Depending on brew time and temperature, a single cup of green tea may contain 100–200 mg EGCG. To control the dose of EGCG administered in experimental studies, green tea solids (GTS) or capsules of green tea extract standardized to EGCG content are often employed [McKay and Blumberg, 2007].

1.10.2.2 Black Tea

About 78% of the consumed tea is black tea, a popular drink in many Western countries, which contains mainly oxidized Polyphenol [Graham, 1992; Mukhtar and Ahmad, 2000]. If the leaves are left to ferment, the leaves are used for black tea. During black tea production, the catechins are converted to theaflavins and thearubigins [Lorenz and Urban, 2009]. In black tea, the polymerized catechins, theaflavins (TF) and thearubigins are found predominately [McKay and Blumberg, 2007].
1.10.2.3 Oolong Tea

The leaves used for oolong tea are only partially fermented [McKay and Blumberg, 2007].

1.10.2.4 White Tea

White tea is made from unopened buds that are fired or steamed before drying, and, like green tea leaves, are not subjected to fermentation [McKay and Blumberg, 2007].

1.10.3 Role of Fermentation

The fermentation is done via the action of Polyphenol oxidase and peroxidase. The fermentation process forms catechin oligmers and high molecular weight complexes of catechins with proteins, caffeine or other leaf ingredients. Post-harvest fermentation alters the relative catechin content of the leaves [McKay and Blumberg, 2007].

1.10.4 Biosynthesis of Tea Flavonoids

The key enzymes in the biosynthesis of tea flavonoids appear to be

(a) 5-dehydroshikimate reductase
(b) phenylalanine ammonia lyase and
(c) Those associated with the shikimate/arogenate pathway [Balentine et al., 1998].

1.11 Resveratrol (3, 5, 4’-trihydroxy-trans-stilbene)

Resveratrol (RES, 3, 5, 4’-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol, and a phytoalexin produced naturally by several plants including grapes [Zivna et al., 2013], mulberries, cranberries, and peanuts when under attack by pathogens such as bacteria or fungi [Melchior and Kindl, 1990; Rayalam et al., 2011; Li et al., 2011, Wang et al., 2013]. Its relatively simple molecular structure enables free radicals overproduced in disease conditions to be scavenged and the redox signaling pathways of the cells to be regulated [Kelkel et al., 2010].
Properties

Resveratrol is a powerful phytoestrogen [Zivna et al., 2013]. The molecule demonstrated wide variety of beneficial effects in cardiovascular diseases, cancers, diabetes, and neurodegenerative disorders [Yu et al., 2012]. Resveratrol is reported to display antitumor activities on a variety of human cancer cells [Boissy et al., 2005]. It is an orally active phytochemical that has many beneficial actions in a variety of animal disease models [Rayalam et al., 2011]. Resveratrol was found to be one of the most active of Polyphenols that directly or indirectly stimulate Sir2 activity (Silent Information Regulator, sirtuin protein) in yeast [Howitz et al., 2003]. The increased NAD concentrations stimulate the activity of Sir2. Sir2, the mammalian homologue of which is known as Sirt1, a NAD+ dependent protein deacetylase, and has been demonstrated to mimic estrogen [He et al., 2010], was shown to modify several proteins that are involved in cellular processes affecting longevity [Rayalam et al., 2011]. It was shown that adding additional copies of the gene coding for the production of Sir2 increased the life span of yeast and Caenorhabditis elegans [Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001].
1.11.1 ‘The French Paradox’

Frenchmen suffer a relatively low incidence of coronary heart disease, despite having a diet relatively rich in saturated fats. The Frenchman’s high red wine consumption is a primary factor for the trend. One of the components of red wine potentially related to this effect is resveratrol [Rayalam et al., 2011].

1.11.2 Resveratrol and osteoporosis

Some natural flavonoids with potent antioxidant activity including scopoletin, resveratrol, and baicalein have found to exert antiosteoporotic activities through suppressing osteoclast formation and TRAP [Morgan et al., 2014]. The bone protective effects of resveratrol have been demonstrated in several osteoporosis models while the underlying mechanism is largely unclear [He et al., 2010]. Resveratrol may inhibit the differentiation and bone resorbing activity of osteoclasts and promote the formation of osteoblasts from mesenchymal precursors in vitro [Kupisiewicz et al., 2010; Zivna et al., 2013]. Resveratrol is found to increase osteoclastic and decreased osteoblastic activities resulting in bone resorption and loss of bone mass [Shakibaei et al., 2011; Zivna et al., 2013]. It is known to increase DNA synthesis and ALP activity in osteoblasts and to prevent femoral bone loss in ovariectomized (OVX) rats [Li et al., 2011]. The incorporation of resveratrol is found to cause increased ALP activity of rat bone marrow stromal cells and enhanced mineralization of the cell–scaffold composites in vitro [Li et al., 2011]. Resveratrol is a naturally occurring phytoestrogen possesses bone-protective effects by antagonizing adipogenesis [Tseng et al., 2011]. Resveratrol promotes osteogenesis of human mesenchymal stem cells (hMSCc) by up regulating RUNX2 gene expression via the SIRT1/FOXO3A axis as a novel mechanism [Tseng et al., 2011]. RES has protective effects on multiple events associated with osteoporosis. Treatment with RES delayed age-related bone loss in rats and mice [Pearson et al., 2008] and protected against bone loss induced by estrogen deficiency [Liu et al., 2005]. Chronic RES supplementation maintained the BMD and strength of the femur of rat hind limb unloading [Momken et al., 2011]. Prior treatment with RES preserved density and structure of rat long bones under tail-suspension [Habold et al., 2011]. Resveratrol at non-toxic concentrations dose-dependently inhibited RANKL-induced osteoclast
differentiation and induced apoptosis of murine osteoclast progenitor RAW 264.7 cells [He et al., 2010]. In cultures of human primary monocytes, resveratrol inhibits dose-dependently RANKL—induced formation of TRACP—positive multinucleated cells, TRACP activity in the medium, up regulation of CTSK gene expression, and bone resorption [Boissy et al., 2005]. Resveratrol promotes dose dependently the expression of osteoblast markers like OCN and OPN in human bone marrow mesenchymal stem cells (hMSC-TERT) and stimulates their response to 1,25(OH)2 vitamin D3 [1,25(OH)2D3] [Boissy et al., 2005]. Resveratrol up-regulates dose-dependently the expression of 1,25(OH)2D3 nuclear receptor [Boissy et al., 2005]. Resveratrol is found to suppress RANKL-induced ROS generation in a concentration dependent manner. The direct inhibitory effects of resveratrol on osteoclastogenesis induced by RANKL in several cell models [Lin et al., 2013], are mediated via inhibition of ROS generation [He et al., 2010]. Resveratrol at non-toxic concentrations dose-dependently is found to inhibit the formation of osteoclasts and the activation of TRAP [Lin et al., 2013]. Resveratrol might inhibit the differentiation of RAW264.7 cells into osteoclasts and decrease osteoclast activation possibly via suppressing monocytes to differentiate preosteoclasts [Lin et al., 2013].

1.12 Garlic

Garlic (Allium sativum L.) is one of the plants that were seriously investigated over several years and used for centuries to fight infectious diseases [Onyeagba et al., 2004; Gebreyohannes and Gebreyohannes, 2013]. Garlic is nicknamed as Russian penicillin for its widespread use as a topical and systemic antimicrobial agent; it is commonly used in many cultures as an excitement and reputation of healing power [Timbo et al., 2006].

1.12.1 Constituents of Garlic

Garlic contains at least 33 sulfur compounds, several enzymes and the minerals germanium, calcium, copper, iron, potassium, magnesium, selenium and zinc; vitamins A, B1 and C, fiber and water. It also contains 17 amino acids to be found in garlic: lysine, histidine, arginine, aspartic acid threonine, swine, glutamine, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tryptophan and
phenylalanine [Josling, 2005]. Whole garlic and aged garlic extract exhibit direct antioxidant effects and enhance the serum levels of two antioxidant enzymes, catalase and GPx [Prasad et al., 1995].

1.12.2 Allicin

Allicin (diallyl thiosulfinate or diallyl disulfide) is one of the most biologically active compounds in garlic. Alliin (S-allylcysteine sulfoxide) is the most abundant sulfur compound in garlic. It is present at 10 and 30 mg/g in fresh and dry garlic, respectively [Lawson, 1998]. Allicin is garlic’s defense mechanism against attacks by pests. Allicin is an organosulfur compound obtained from garlic, a species in the family Alliaceae [Block, 1985]. Allicin is efficiently scavenged exogenously generated hydroxyl radicals in a dose dependent fashion, but their effectiveness was reduced about 10% by heating to 100 °C for 20 min [Gebreyohannes and Gebreyohannes, 2013].

1.12.2.1 Structure of Allicin

Allicin features the thiosulfinate functional group, R-S-(O)-S-R. The compound is not present in garlic unless tissue damage occurs. Typical garlic food preparation such as chopping, mincing and crushing disturbs S-allyl cysteine sulfoxide and exposed it to the allinase enzymes, then quickly converted it to diallyl thiosulfinate, which give off garlic’s characteristic aroma. The allinase enzyme responsible for diallyl thiosulfinate conversion becomes inactivated below a pH of 3.5 or with heating [Block, 1985; Pedrazza-Chaverri et al., 2006]. Allicin is chiral but occurs naturally only as a racemate [Block, 2010]. The racemic form can also be generated by oxidation of diallyl disulfide [Cremlyn, 1996].

\[
(SCH_2CH=CH_2)_2 + RCO_3H \rightarrow CH_2=CHCH_S(O)SCH_2CH=CH_2 + RCO_2H
\]

Alliinase is irreversibly deactivated below pH 3. Allicin is generally not produced in the body from the consumption of fresh or powdered garlic [Brodnitz et al., 1971; Yu and Wu, 1989]. Allicin can be unstable, breaking down within 16 h at 23 °C [Hahn, 1996].
1.13 Turmeric

Turmeric is the popular South Asian dietary spice, which is a member of the ginger family (Zingiberaceae).

1.13.1 Curcuminoids

Turmeric has three curcuminoids:

(a) Curcumin
(b) Desmethoxycurcumin
(c) bis-desmethoxycurcumin

The curcuminoids are natural phenols that are responsible for the yellow color of turmeric.

1.13.2 Curcumin

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-hepadiene-3,5-dion or diferuloylmethane] is a hydrophobic polyphenolic compound or pigment derived from turmeric [Kolev et al., 2005]. Curcumin is known to be powerful antioxidant [Moon et al., 2012]. The antioxidant activity of curcumin arises from scavenging of several biologically free radicals that are produced during physiological processes and possesses several pharmacological effects including anti-inflammatory, antioxidant, antiproliferative, and antiangiogenic activities [Aggarwal et al., 2003]. Curcumin is
the principal curcuminoid of Turmeric. Curcumin can exist in several tautomeric forms, including a 1,3-diketo form and two equivalent enol forms. The main drawback associated with the therapeutic potential of curcumin is its poor aqueous solubility and stability, which leads to poor bioavailability [Aggarwal et al., 2003]. The enol form is more energetically stable in the solid phase and in solution [Kelev et al., 2005].

**Figure XXV (a): Structure of Curcumin (Enol Form), source**
http://en.wikipedia.org/wiki/File:Curcumin.svg

**Figure XXV (b): Structure of Curcumin (Keto Form), source**

### 1.13.3 Curcumin and Osteoporosis

Curcumin is a potent inhibitor of the transcriptional factors activator protein-1 and NF-κB [Ozaki et al., 2000]. Curcumin is found to be a potent stimulator of apoptosis process in rabbit osteoclasts, as evidenced by morphological changes in nuclei and DNA fragmentation as criteria of apoptosis, in a dose-and treatment time-dependent manner [Ozaki et al., 2000]. Curcumin is found to have the ability to suppress RANKL signaling and osteoclastogenesis in RAW 264.7 cells, a murine monocytic cell line [Bharti et al., 2004]. Curcumin is found to inhibit the pathway leading from activation of IκBα kinase and IκBα phosphorylation to IκBα degradation [Bharti et al., 2004]. RANKL is found to induce osteoclastogenesis in these monocytic cells, and curcumin is found to inhibit both RANKL- and TNF-induced osteoclastogenesis and pit formation [Bharti et al., 2004]. Curcumin suppressed osteoclastogenesis maximally when added together with RANKL and minimally when it was added 2
days after RANKL [Bharti et al., 2004]. Curcumin is found to markedly inhibit the formation of TRAP-positive multinucleated cells in both bone marrow-derived monocytes (BMMs) and RAW 264.7 cells [Moon et al., 2012]. Curcumin is found to scavenge intracellular ROS generation within osteoclast precursors during RANKL-stimulated osteoclastogenesis [Moon et al., 2012]. Curcumin is also found to significantly suppress the gene expression of NFATc1, TRAP, and osteoclast-associated immunoglobulin-like receptor (OSCAR), which are genetic markers of osteoclast differentiation in a dose-dependent manner [Moon et al., 2012]. Curcumin is found to display the highest inhibitory effect on osteoclast differentiation when concentrations were held constant [Moon et al., 2012]. Curcumin together with CoQ10 and selenite, is found to act as inhibitor of RANKL-induced NFATc1 through suppression of ROS generation, suggesting the potential usefulness for the treatment of bone disease associated with excessive bone resorption [Moon et al., 2012]. Most recently, curcumin has been reported to inhibit prostate cancer bone metastasis by up-regulating bone morphogenic protein-7 (BMP-7) in vivo [Dorai et al., 2014].

Therefore, in view of the above, there is an immediate need to develop new cost-effective therapeutic strategies against osteoporosis which will help in improved survival as well as in combating the increase in the age-specific fracture rates. Thus, in the present study, resveratrol, curcumin as well as allicin from garlic were employed as valuable natural antioxidants / natural tools in order to investigate the above, which in turn, may prove beneficial in the management of osteoporosis.