2 REVIEW

Bone is a highly mineralized connective tissue that together with cartilage forms the skeletal system and provides both structural and metabolic functions. The adult human skeleton consists of 206 bones, excluding the sesamoid bones. The appendicular skeleton has 126 bones, axial skeleton 74 bones, and auditory ossicles six bones (Clarke, 2008). It is dynamically characterized by its rigidity, hardness, and power of regeneration and repair with some degree of elasticity. Bones exert important functions including structural support, movement and locomotion by acting as levers for the muscle, protect vital internal organs, maintain mineral homeostasis (especially mineral reservoir for calcium equilibrium), take part in acid–base balance, serve as a reservoir of growth factors and cytokines and provide the environment for hematopoiesis within the marrow spaces both blood forming and fat storage (Taichman, 2005). All the bones are in the constant processes of modeling (reshaping) and remodeling throughout the life, to help in adapting the changes of biomechanical forces and hormonal stimuli, as well as remodeling to remove old, microdamaged bone and replace it with new, mechanically stronger bone. Constant removal and renewal helps to preserve the bone strength (Kini & Nandeesh, 2012).

Anatomically, bones can be classified under four categories: long bones, short bones, flat bones, and irregular bones. Long bones including clavicles, humeri, radii, ulnae, metacarpals, femurs, tibiae, fibulae, metatarsals, and phalanges; Short bones - carpal and tarsal bones, patellae, and sesamoid bones; Flat bones - skull, mandible, scapulae, sternum, and ribs; Irregular bones include the vertebrae, sacrum, coccyx, and hyoid bone. Flat bones are formed by membranous bone formation, whereas long bones are made by the combination of endochondral and membranous bone formation (Matsumoto et al., 2005; Saito et al., 2010). Bone is composed by organic matrix and inorganic salts in the form of small crystals. The organic matrix consists of 90 % of Type I collagen, and noncollagenous proteins including osteocalcin, osteonectin, osteopontin, fibronectin and bone sialoprotein II, bone morphogenetic proteins (BMPs), and growth factors (Aszódi, Bateman, Gustafsson, Boot-Handford, & Fässler, 2000). Calcium and phosphate ions nucleate to form the hydroxyapatite crystals\[Ca_{10}(PO_4 )_6(OH)_{2}\], with small amounts of carbonate, magnesium, and acid phosphate (Datta, Ng, Walker, Tuck, & Varanasi, 2008). It is generally composed of 50 to 70% mineral, 20 to 40% organic matrix, 5 to 10% water, and <3% lipids.
Histologically a mature bone has 2 components - the cortical or compact bone is a dense, solid, and that surrounds the marrow space whereas trabecular bone is composed of a honeycomb-like network of trabecular plates and rods interspersed in the bone marrow compartment. Trabecular bone also known as spongy or cancellous bone as that resembles a sponge. An 80% of the adult human skeleton is composed of cortical bone and 20% trabecular bone overall. Different bones and skeletal sites may have different ratios of cortical to trabecular bone. The vertebra is composed of cortical to cancellous bone in a ratio of 1:3, femoral head has 50:50 and radial diaphysis consist of 95% cortical and 5% cancellous bone (Eriksen, Axelrod, & Melsen, 1994). Cortical or compact bone is found mainly in the shaft of long bones and the surfaces of flat bones, whereas trabecular or cancellous bone being less in number occupies the ends of these bones.

**Structure of Long Bone**

![Structure of Long Bone](image)

Fig 4 Adapted from Pearson Education, Inc., publishing as Benjamin Cummings 2006

Cortical bone has an outer periosteal surface and inner endosteal surface. The outer cortical surface of bone is surrounded by fibrous connective of periosteum, except at joints where bone is lined by articular cartilage. Periosteum contains blood vessels, nerve fibers, osteoblasts, and osteoclasts. Periosteal surface activity has important role in appositional
growth and fracture repair and hence, protects, nourishes and aides in bone formation (Eriksen et al., 1994). The endosteam is a membranous structure covering the inner surface of cortical and cancellous bone and the blood vessel canals (Volkmann’s canals) present in bone. Both cortical and trabecular bone are composed of lamellations of collagenous fibers that appear as stacks of parallel or concentrically arranged layers (Shea & Miller, 2005). In cortical bone the lamellae are concentrically arranged around a vascular canal (Haversian canal) and collectively called an osteon or Haversian system whereas in trabecular bone they are parallel to one another (Datta et al., 2008). Haversian systems are cylindrical in shape, are approximately 400 mm long and 200 mm wide at their base. A healthy human adult have approximately 21×10^6 cortical and 14×10^6 trabecular osteons with a total Haversian remodeling area of approximately 3.5m^2 and 7m^2 in cortical and cancellous bones respectively.

Additionally, trabecular bone is highly metabolic tissue than the cortical bone as it possess more surface area and act as the repository of bone minerals such as calcium, magnesium and phosphorus. Woven bone is formed by osteoblasts during the osteoid production as a irregular collagen fibers structure. This initially occurs in all fetal bones during embryonic growth and in fracture healing, but later is replaced with mature lamellar bone with orderly deposition of the collagen fibers in a highly organized parallel or concentric layered structure by a remodeling process (Clarke, 2008).

2.1 PHYSIOLOGY OF BONE FORMATION

Bone is composed of four different cell types - Osteoblasts, osteoclasts, and bone lining cells are present on bone surfaces, whereas osteocytes permeate the mineralized interior.

2.1.1 OSTEOBLASTS

Osteoblasts originate from pluripotent mesenchymal stem cells (MSC) of the bone marrow stroma that have the potential to differentiate into adipocytes, myocytes, chondrocytes and osteoblasts under the direction of a defined suite of regulatory transcription factors. Osteoblasts are mononucleated, vary from flat to plump in shape that depends upon their cellular activity and maturity stage. Mature active osteoblast are cuboidal cells that are located along the bone surface occupying 4–6% of the total resident bone cells (Capulli, Paone, & Rucci, 2014). Morphologically, these cells decipher abundant rough endoplasmic
reticulum, prominent Golgi apparatus and various secretory vesicles of protein synthesizing machinery (Marks & Popoff, 1988).

Commitment of MSC towards the osteoprogenitor lineage requires the expression of specific genes, members of the canonical Wingless (Wnt)/βcatenin pathways and associated proteins such as bone morphogenetic proteins (BMPs) (Grigoriadis, Heersche, & Aubin, 1988; Logan & Nusse, 2004). The transcription and expression of Runt-related transcription factors 2 (Cbfa1-core-binding factor A1/Runx2), Distal-less homeobox 5 (Dlx5), and Osterix (Osx), a downstream factor of Runx2 are crucial requirements for the osteoblast differentiation (Capulli et al., 2014; Ducy, Zhang, Geoffroy, Ridall, & Karsenty, 1997). Additionally, it has been demonstrated that Runx2 is a master gene of osteoblast differentiation by the fact that Runx2- null mice were completely lack mineralized tissue due to arrest of osteoblast maturation (Komori et al., 1997). In human, mutations in Runx2 cause Cleido Cranial Dysplasia (CCD), an autosomal dominant disease with dramatic abnormalities in the bones is formed by intramembranous ossification in humans (Lee et al., 1997). Further, it is manifested the role of transcription factor Runx2 in the upregulation of osteoblast-related genes such as CollA1, ALP, BSP, BGLAP and OCN in osteoblasts as well as in non-osteoblastic cells, such as fibroblasts (Fakhry, Hamade, Badran, Buchet, & Magne, 2013). Osteoblasts are responsible to regulate the differentiation and activity of the bone-resorbing osteoclasts and bone matrix deposition (Mackie, 2003).

![Fig 5 Adapted from Clinical Cases in Mineral and Bone Metabolism 2008.](image)

The active osteoblasts are highly enriched in ALP (alkaline phosphatase, an organic phosphate-splitting enzyme) and secrete bone matrix proteins such as collagen Type I, several non-collagenous proteins such as osteocalcin, osteopontin, osteonectin and bone
sialoprotein II (BSP II). ALP and the type 1 parathyroid receptor (PTH1R) are early markers of osteoblast progenitors that increase as osteoblasts mature and deposit matrix. Finally, their number get reduced as some osteoblasts become trapped in their own bone matrix, giving rise to osteocytes which, gradually, stop secreting osteoid. The level of osteocalcin (late marker) is upregulated in post-proliferative mature osteoblasts associated with mineralized osteoid (Glass II & Karsenty, 2007).

2.1.2 OSTEOCLASTS

Osteoclasts are giant, terminally differentiated multinucleated cells, tartrate-resistant acid phosphatase (TRAP)-positive cells, which are derived from mononuclear cells of the monocyte/macrophage lineage, under the influence of several factors. Two hematopoietic cytokines, macrophage-colony stimulating factor (M-CSF), secreted by osteoprogenitor mesenchymal cells and receptor activator of nuclear factor κβ ligand (RANKL), secreted by osteoblasts, osteocytes, and stromal cells are crucial for the differentiation of macrophage precursors into mature osteoclasts. Osteoclasts contain abundant Golgi complexes, mitochondria and transport vesicles loaded with lysosomal enzymes. Functionally, M-CSF binds to its receptor (cFMS) present in osteoclast precursors, which initiate the proliferative and differentiation phase of osteoclast development as well osteoclast survival and cytoskeletal rearrangement. Binding of RANKL with its receptor RANK, a transmembrane receptor, belongs to the tumour necrosis factor (TNF) receptor superfamily and is expressed on the surface of preosteoclasts and mature osteoclasts, is crucial for osteoclastogenesis and bone resorption (Kong et al., 1999; J. Li et al., 2000).

Alternatively, Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL, produced by a wide range of cells including osteoblasts, stromal cells, and gingival and periodontal fibroblasts (Longhini, Oliveira, Sasso-cerri, & Cerri, 2014), binds to RANKL, preventing the RANK/RANKL interaction. Consequently, blocking the osteoclast formation in vitro within the bone environment and bone resorption in vivo reducing its ability to interact RANK (Boyce & Xing, 2008). The earlier events in the bone resorption is the production of hydrogen ions and cathepsin K enzyme from activated osteoclast leading to bone resorption. Released protons acidify the resorption compartment beneath osteoclasts to dissolve the mineral component of bone matrix, whereas cathepsin K digests the proteinaceous matrix, which is mainly composed of type I collagen (Boyle, Simonet, & Lacey, 2003).
2.1.3 OSTEOCYTES

Osteocytes are derived from MSCs lineage through osteoblast differentiation. They are represented as terminally differentiated osteoblasts and function within syncytial networks to support bone structure and metabolism. Osteocytes comprise of 90–95% of the total bone cells, and are the most abundant and long-lived cells, with a lifespan of up to 25 years (Franz-Odendaal, Hall, & Witten, 2006). They are located within lacunae surrounded by mineralized bone matrix and have extensive filipodial processes similar to dendritic morphology, that lie within the canaliculi in mineralized bone (Bonewald, 1999). Generally, these cells do not express alkaline phosphatase but do express osteocalcin, galectin 3, and CD44, a cell adhesion receptor for hyaluronate, and several other bone matrix proteins that support intercellular adhesion and regulate exchange of mineral in the bone fluid within lacunae and the canalicular network. During osteolysis, they become active and may function as phagocytic cells.

Osteocytes are metabolically and electrically linked through gap junctions composed primarily of connexin43 which is required for osteocyte maturation, activity, and survival (Plotkin, Manolagas, & Bellido, 2002). Mechanosensitive function of osteocytes is to transduce stress signals from bending or stretching of bone into biologic activity, a phenomenon that is called piezoelectric effect (Rubin & Lanyon, 1987). It has been suggested that presence of empty lacunae in aging bone is due to the osteocytes which may undergo apoptosis, probably caused by disruption of their intercellular gap junctions or cell–matrix
interactions (Xing & Boyce, 2005). Osteocyte apoptosis in response to estrogen deficiency or glucocorticoid treatment is harmful to bone structure which can be prevented by estrogen and bisphosphonate therapy and physiologic loading of bone (Plotkin, Aguirre, Kousteni, Manolagas, & Bellido, 2005).

2.1.4 BONE LINING CELLS

Bone lining cells are quiescent, thin, elongated osteoblasts that cover most bone surfaces in the mature skeleton, where neither bone resorption nor bone formation occurs (Miller, Saint-Georges, Bowman, & Jee, 1989). These cells exhibit cytoplasmic extensions or gap junctions that connect them to the adjacent bone lining cells and between these cells and osteocytes. As they are metabolically inactive cells they contain fewer organelles and less cytoplasm than osteoblasts. Researchers are still speculating the function, as these cells have shown to prevent the direct interaction between osteoclasts and bone matrix, where bone resorption should not occur, and also participate in osteoclast differentiation, producing osteoprotegerin and the receptor activator of nuclear factor kappa-B ligand (Andersen et al., 2009). Further, together with other bone cells, bone lining cells, constitute an important component of the BMU (basic multicellular units), an anatomical structure that is present during the bone remodeling cycle (Everts et al., 2002).

2.2 BONE MODELING AND REMODELING

During the entire life, bone undergoes longitudinal and radial growth, modeling, and remodeling. Longitudinal and radial growth are associated with childhood and adolescence growth and development phase, at the site of growth plates, where cartilage proliferates in the epiphyseal and metaphyseal areas of long bones, before subsequently undergoing mineralization to form primary new bone.

2.2.1 BONE MODELING

Modeling is the reshaping process through which bones change their overall shape in physiologic influences or mechanical forces, resulting in gradual adjustment of the skeleton to the forces. In response to biomechanical forces, bones may widen or change their axis by removal or addition of bone to the appropriate surfaces by independent action of osteoblasts and osteoclasts. The function is similar during aging in response to periosteal apposition of new bone and endosteal resorption of old bone. Bone Modeling is less frequent in adults than
remodeling (Kobayashi et al., 2003) and may get enhanced in hypoparathyroidism (Ubara et al., 2005), renal osteodystrophy (Ubara et al., 2003), or treatment with anabolic agents (Lindsay et al., 2006).

2.2.2 BONE REMODELING

Bone remodeling is a lifelong physiological process that involves continuous removal of discrete packets of old bone from the skeleton (a subprocess called bone resorption) and replacement with newly synthesized proteinaceous matrix (a subprocess called ossification or bone formation), further with subsequent mineralization of the matrix to form new bone (Hernández-Gil, Gracia, Pingarrón, & Jerez, 2006). These processes maintain the reshaping or replacement of bone during growth, microfracture, mechanical stress, provide bone strength, structural integrity to the skeleton and mineral homeostasis (C. H. Turner, 1998).

Although cortical bone shares 75% of the total volume, the metabolic rate is 10 times higher in trabecular bone since the surface area to volume ratio is much greater (trabecular bone surface representing 60% of the total). Therefore, approximately 5 to 10% of total bone is renewed per year.

Remodeling rate increases in perimenopausal and early postmenopausal women and then slows with further aging but continues at a faster rate than in premenopausal women. At the microscopic level, bone remodeling takes place in bone cavities, as basic multicellular units (BMU), comprising of a group of osteoclasts ahead forming the cutting cone and a group of osteoblasts behind forming the closing cone, associated with blood vessels and the peripheral innervations (Elefteriou, 2008; Matsuo & Irie, 2008). Bone remodelling is accomplished by sequential phases of these processes: activation, resorption, reversal and formation.

a) Activation phase

Microfracture, mechanical loading alternations and release of some factors including insulin growth factor-I (IGFI), tumour necrosis factor-α (TNF-α), parathyroid hormone (PTH) and interleukin-6 (IL-6), in the bone microenvironment, trigger the activation of the lining cells that are inactive osteoblasts. As a result, there is increased surface expression of RANKL on the lining cells, which in turn communicates with its receptor RANK, expressed by pre-osteoclasts. As a consequence, of RANKL/ RANK interaction, triggers pre-osteoclasts fusion and differentiation toward multinucleated osteoclasts.
b) Resorption phase

After differentiation, osteoclast becomes polarized and gets adhered to the bone surface and begins to dissolve bone matrix. These resorbing osteoclasts start secreting hydrogen ions via $\text{H}^+$-ATPase proton pumps and chloride channels in their cell membranes into the resorbing compartment to lower the pH to as low as 4.5 within the bone resorbing compartment, which helps to mobilize bone mineral. They also release tartrate resistant acid phosphatase, cathepsin K, matrix metalloproteinase 9 and gelatinase from cytoplasmic lysosomes (Delaissé et al., 2003) which digest the organic matrix, resulting in formation of saucer shaped Howship's lacunae on the surface of trabecular bone and Haversian canals in cortical bone. Once the function is accomplished, these osteoclasts undergo apoptosis, as a physiological consequence to avoid an excessive bone resorption (S. Reddy, 2004).

c) Reverse phase

At the end of resorption phase, the resorption cavities contain a variety of mononuclear cells, including monocytes, osteocytes released from bone matrix and preosteoblasts. The coupling signals connecting the transition of bone resorption to the genesis of bone formation are still unknown, but proposed coupling signal candidates include bone matrix derived factors such as TGF-β, IGF1, IGF2, bone morphogenetic proteins, PDGF, or fibroblast growth factor (Hock, Centrella, & Canalis, 1988; Locklin, Oreffo, & Triffitt, 1999) and strain gradient in the lacunae (Smit, Burger, & Huyghe, 2002). The strain get reduced in front and increased behind, and in Howship's lacunae, whereas it is highest at the base and less in surrounding bone at the edges of the lacunae, in a cutting cone. As a consequence, may lead to sequential activation of osteoclasts and osteoblasts, with osteoclasts activated by reduced strain and osteoblasts by increased strain (Martin & Sims, 2005).

d) Formation phase

Simultaneously in the resorbed areas, with the release of growth factors from the matrix, which act as chemotactics, preosteoblast grouping phenomena is initiated and stimulated for their proliferation (Lind et al., 1995). The preosteoblasts secrete a cementing substance for the attachment of new tissues and expression of bone morphogenic proteins (BMP). Once recruited, the differentiated osteoblasts synthesize new bone matrix, initially not calcified (osteoid), that fills the perforated areas and then promote its mineralization, thus completing the bone remodeling process. Instability between the resorption and formation phases mirror
an incorrect bone remodeling, affecting the bone mass, eventually leading to a pathological condition.

2.3 REGULATORY FACTORS IN BONE REMODELING

The balance between bone resorption and formation is influenced by such interrelated factors as genetic, mechanical, vascular, nutritional, hormonal and local.

2.3.1 SYSTEMIC REGULATION

a) Genetic Factors

Genetic factors determine 60 to 80% of the bone mass. Thus, Negroes have a greater bone mass than Whites, who in turn have a higher mass than Asians. Therefore, daughters of osteoporotic mothers are more predisposed to have this condition themselves (Grant & Ralston, 1997).

b) Mechanical Factors

Physical activity is vital for the correct bone development. Tension transmitted by muscular action to bone is sensed by osteocyte network within the osseous fluid, which further release factors such as prostaglandins, nitric oxide and IGF-I, to stimulate osteoblast activity and
enhanced bone formation. Additionally, absence of muscular activity, rest or weightlessness has an adverse effect on bone by accelerating resorption (Hernández-Gil et al., 2006).

c) Vascular / Nerve Factors

Vascularization is crucial for normal bone development, delivering blood cells, oxygen, minerals, ions, glucose, hormones, and growth factors. It also contributed the first phase in ossification, repair of fracture and bone regeneration (Trueta, 1963). Innervation is fundamental process for normal bone physiology as bone is innervated by the autonomous nervous system and sensory nerve fibers.

d) Nutritional Factors

Minimum dietary calcium recommended for proper bone mineralization is 1200mg per day for 25 age, 1000mg per day from 25 to 45, and following menopause should be at least 1500mg per day. However, toxic habits such as smoking, caffeine, alcohol and excess salt consumption constitute risk factors for osteopenia.

e) Hormonal Factors

Fundamentally, normal skeleton development is conditioned by proper functioning of the endocrine system, growth hormone (GH) and the calcitropic hormones (parathyroid hormone, calcitonin, and metabolites of vitamin D). They act as systemic messengers that have endocrine effect and also regulate synthesis and action of local factors, directly intervening the cellular metabolism.

f) Thyroid hormones

Thyroid hormones have conflicting actions on bone, one by promoting IGF-I synthesis that stimulates osteoid matrix by the osteoblasts and its mineralization. Consequently, producing short stature by altered bone formation in congenital hypothyroidism. Contrary, in hyperthyroidism there is increased bone loss due to enhanced bone resorption by osteoclast activity induced by thyroid hormones (Gimeno et al., 1997).

g) Parathyroid hormone (PTH)

PTH is an important regulator of calcium homeostasis, acting directly on bone and kidney, and indirectly on intestine. It is produced by the parathyroid glands in response to
hypocalcemia. This hormone has dual effect on bone resorption and formation. At high levels, PTH stimulate bone resorption by increased expression of RANKL on osteoblastic cells, favoring osteoclastogenesis, while at intermittent doses it promotes bone formation by increased production of IGF-I and TGF-β growth factors and reduction in osteoblasts apoptosis (E Canalis, McCarthy, & Centrella, 1989).

h) Calcitonin and 1,25 (OH)2 Vitamin D3 or Calcitriol

Calcitonin is produced by the parafollicular C cells of the thyroid, acts as bone resorption inhibitor. However, its action is transitory as the osteoclasts become ‘impermeable’ to calcitonin within a few days (Hernández-Gil et al., 2006). Vitamin D3 is a steroid hormone that favors the intestinal absorption of calcium and phosphate, and therefore promotes bone mineralization that is necessary for normal skeleton growth. It may also be produced by lymphocytic or monocytic bone cells, and hence play an important role as a local regulator of osteoclast differentiation (L. Raisz, 1993).

i) Other Hormones

Androgens have an anabolic effect on bone through the stimulation of the osteoblast receptors. Likewise, they act as mediators of the peak GH in puberty, while androgen deficiency is associated with lower bone density at adult age. Estrogen plays an important role in the bone development of both masculine and feminine, during adolescence. It has a dual effect on bone metabolism, by promoting bone formation and inhibiting resorption. Estrogens upregulate the osteoprotegerin (OPG) levels, a protein produced by osteoblasts, decrease the responsiveness of the osteoclast progenitor cells to RANKL, thereby down regulate osteoclastogenesis (L. Hofbauer et al., 1999). Therefore, during menopause estrogen deficiency lead to pathogenic condition associated with bone loss and osteoporosis. Insulin increase the hepatic synthesis of IGF-I (insulin-like growth factor) which further stimulates matrix synthesis both directly and indirectly.

j) Glucocorticoids

Glucocorticoids have catabolic effect on bone at higher concentrations, by directly suppressing BMP-2 and Cbfa1, critical factors in osteoblastogenesis and inhibiting the synthesis of IGF-I by osteoblasts (Manolagas, 2000). However, at physiological concentrations they have an osteogenic capacity (Lukert & Kream, 1996).
2.3.2 LOCAL REGULATORS

Bone remodeling is also regulated by local factors, such as growth factors and cytokines, and also the bone matrix proteins that are modulators of other local factors (Hernández-Gil et al., 2006; L. G. Raisz, 1999).

a) Growth Factors

IGF-I and II (insulin-like growth factor I and II are polypeptides similar to insulin; synthesized by liver and osteoblasts. They are abundantly found in the osteoid matrix and actively participate in bone modeling (Hill, Reynolds, & Meikle, 1995). IGF synthesis is upregulated by growth hormones, estrogens and progesterone while inhibited by glucocorticoids. IGF-II is primarily important during embryogenesis, but its effects on the fully developed skeleton are as yet unknown (Mohan & Baylink, 1991).

Transforming Growth Factor-β (TGF-β) is second abundant proteins in bone tissue present in the matrix and activate during osteoclastic resorption. TGF-β promotes bone formation by stimulating osteoblastic differentiation and the synthesis of the osteoid matrix, and inhibiting the synthesis of the proteases (especially the matrix metalloproteinase (MMP). It also inhibits the formation and differentiation of osteoclast and stimulate osteoclast apoptosis (Baylink, Finkelman, & Mohan, 1993). Bone morphogenetic proteins (BMP) are included in the TGF-β family. They stimulate the stem cells differentiation towards different cell lines (adipose tissue, cartilage, and bone).

Functionally, they are the strong candidate for the osteoblastic differentiation (Ernesto Canalis, Economides, & Gazzerro, 2003) and also participate during embryogenesis in the formation of bone and cartilage (Yamaguchii, Suda, & Komori, 2000). Platelet - derived growth factor (PDGF) and Fibroblastic growth factor (FGF) also stimulate bone formation whereas Epidermal growth factor (EGF), Vascular endothelial growth factor (VEGF), Granulocyte / macrophage - colony stimulating factor (GM-CSF), Macrophage-colony stimulating factor (M-CSF), Tumor Necrosis Factor (TNF) stimulate bone resorption.

b) Cytokine

Cytokines are the polypeptides synthesized in the lymphocytic and monocytic cells and have multiple cellular functions, such as the immunological response, inflammation and
hematopoiesis. Interleukin 1 (IL-1) stimulates the osteoclastic resorption, by increasing the proliferation and differentiation of the pre-osteoblasts and osteoclastic activity and inhibiting osteoclasts apoptosis of the osteoclasts (Compston, 2001). Interleukin 6 (IL-6), Interleukin 11 (IL-11), Prostaglandins (PG) and Leukotrienes stimulate bone resorption (Kini & Nandeesh, 2012).

2.4 OSTEOPOROSIS

The word osteoporosis is derived from the Greek language; ‘osteo’ meaning bone, and ‘poros’ meaning porous. The terminology itself gives an instant impression of the disorder and allows one to envisage its implications. Osteoporosis, a major public health problem, is characterized by a reduction in and deterioration of the micro-architecture of bone tissue, with a consequent increase in bone frailty and susceptibility to fractures (Zhao et al., 2011). It is a skeletal disorder characterized by a reduction in bone strength which predisposes to increased fracture risk. However, low BMD confers increased risk for fracture, most fractures occur in postmenopausal women and elderly men (Pasco et al., 2006). Osteoporosis has become a serious health hazard taking a huge personal and economic toll. It has been estimated that in Europe, the disability due to osteoporosis is greater than that caused by cancers (with the exception of lung cancer) and is comparable or greater than that lost to a variety of chronic noncommunicable diseases, such as rheumatoid arthritis, asthma and high blood pressure related heart disease (Johnell & Kanis, 2006).

2.5 DIAGNOSIS

2.5.1 Bone Mineral Density Testing Modalities

Varieties of diagnostic techniques have been utilized for the determination of bone density in the diagnosis of osteoporosis, fracture risk, and monitoring progress of pharmacotherapy. Several radiographic methods such as Single-energy X-ray absorptiometry (SXA) and dual-energy X-ray absorptiometry (DXA or DEXA) are used for measuring the BMD and mineral content of the entire skeleton as well as of specific sites. DEXA is the most accepted gold standard for diagnosis and correlation of fracture risk at the hip (femoral neck) and lumbar spine because it has low radiation exposure, fast, and precise measurements at the spine, hip, radius, or other peripheral site (Tucci, 2006). Quantitative ultrasound (QUS), measures speed and attenuation of sound in bone to evaluate bone density, is more portable, economical, and does not use ionizing radiation, can be used to assess fracture risk. However, the screening
accuracy of QUS is less than DEXA at predicting fracture risk, therefore, it is recommended that abnormal BMD results on QUS be confirmed by DEXA (Lewiecki, Richmond, & Miller, 2006).

With the advent of quantitative computed tomography (QCT), the assessment of spine and appendicular skeleton has become convenient for the accurate measurement of bone density. The technique is suitable for monitoring response to treatment since it measures the cancellous bone that is more responsive to treatment. Although QCT provides information on the shape and bone macroarchitecture, its major disadvantages are high radiation exposure, difficulties with quality control and high relative cost (World Health Organization, 2003). The National Institute for Health and Care Excellence (NICE) has recommended the use of Fracture prediction calculators, including FRAX® (Fracture Risk Assessment), QFractureScores®, and the Garvan Institute fracture calculator along with DEXA scan, as algorithm-based methods to estimate fracture risk, considering lifestyle factors such as body mass index, alcohol and smoking history; to provide a fracture prediction score in all women age 65 years or older and men age 75 years or older (National Institute for Health and Care Excellence. NICE Clinical Guideline 146., 2012).

2.5.2 BONE TURNOVER MARKERS

Biochemical markers of bone metabolism are the products of active osteoblasts that are expressed during different phases of their development and related to different aspects of osteoblast function and bone formation. These biomarkers can be estimated in serum, plasma and urine.

a) Bone Formation Markers

Serum Alkaline Phosphatase (ALP) is a ubiquitous, membrane-bound tetrameric enzyme found in the plasma membrane of the osteoblasts and other cells. It has important role in osteoid formation and mineralization at an alkaline pH. Serum alkaline phosphatase activity is the most commonly used bone formation marker in both clinical and research evaluation. Various isoforms of ALP have been identified in liver, intestine, placenta and bone. Further, there is dissimilarity among the isoenzymes as the hepatic isoenzyme is heat stable, whereas bone origin is thermolabile (Delmas, 1993). Osteocalcin, also known as bone GLA protein (BGP), is a hydroxyapatite-binding protein exclusively synthesized by osteoblasts,
odontoblasts and hypertrophic chondrocytes. It constitutes 15% of the noncollagenous bone matrix and proven sensitive and specific marker of osteoblast activity in variety of metabolic bone diseases. Osteocalcin functioning is dependent upon the vitamin D metabolites, especially 1,25-dihydroxyvitamin D and vitamin K for the γ carboxylation of its three glutamate residues to gamma-carboxyglutamate (gla). Partial or incompletely carboxylated osteocalcin have been majorly found in osteoporotic elderly patients. It has been a useful biomarker in steroid-induced Osteoporosis (Shetty, Kapoor, Bondu, Thomas, & Paul, 2016).

Type I collagen is the major structural protein of bone that constitutes 90% of the organic material and provides structural integrity and strength to the matrix. During the collagen synthesis, procollagen Type I N-terminal propeptide (PINP) and procollagen type I C-terminal propeptide (PICP) peptides are released after posttranslational cleavage of type I procollagen molecules by proteases at N- and C-terminal, respectively. They predominantly originate from proliferating osteoblasts and fibroblasts with small contributions from skin, tendon, dentin, and cartilage. The occupancy of PINP and PICP in the serum or urine is the indication of the osteoblastic bone formation (Garnero, Vergnaud, & Hoyle, 2008).

b) Bone Resorption Markers

Bone resorption markers are the byproducts of osteoclasts activity released during bone resorption of bone remodeling. Carboxy and amino terminal cross linked Telopeptides of Type I Collagen (CTX and NTX) are released in the urine and serum as cleavage products of Type I collagen by cathepsin K during the resorption phase of bone turnover. NTX levels are altered in liver and renal failure. During bone resorption, Type I collagen is proteolytically cleaved to release covalent crosslinks pyridinium compounds - pyridinoline [PYD] and deoxypyridinoline (DPD). Their levels strictly reflect the degradation of mature, i.e. crosslinked collagens so not influenced by the degradation of newly synthesized collagens.

The occurrence of PYD mainly found in cartilage, bone, ligaments, and vessels, whereas DPD is restricted to bone and dentin. Currently, urinary pyridinium crosslinks have been used as promising markers to measure bone resorption (Delmas, 1993). Acid phosphatase is a lysosomal enzyme that exists in several forms in different tissues. Tartrate-resistant acid phosphatase isoform 5b (TRAP5b) are produced by osteoclast and is involved in the degradation of collagen matrix. Serum TRAP level is a good indicator for the
osteoclastic bone resorption typically associated with increased bone turnover conditions, such as Paget’s disease, bone metastases, multiple myeloma, and after ovariectomy (Halleen et al., 2000). Cathepsin K is a cysteine proteinase expressed and secreted by osteoclasts during active bone resorption and also a specific biochemical marker of osteoclastic activity.

Hydroxyproline (Hyp) is a modified amino acid derived from proline (Pro) by hydroxylation in collagen biosynthesis. It is the mainly found collagens, where its hydroxyl groups assist stabilization of the collagen fiber. Peptides and hydroxyproline are released as byproducts of collagen breakdown by collagenase into serum and urine and cannot be reutilized for collagen biosynthesis. Therefore, urinary hydroxyproline has an important role in assessing bone resorption and collagen metabolism related disorders (Neuman & Logan, 1950). However, nowadays it is no longer widely used due to lack of specificity for bone resorption because excreted hydroxyproline also comes from other tissues, particularly from skin collagen, from newly synthesized collagen that is not incorporated into tissue, and from dietary collagen and gelatin.

2.6 OSTEOPOROSIS MANAGEMENT

The pharmacological intervention primarily aims to reduce risk of fracture either by suppression of bone resorption with antiresorptive treatment, or stimulation of bone formation with anabolic agents. Each drug has been approved by the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA). In the case of strontium ranelate, administered with calcium and vitamin D supplementation.

2.6.1 ANTIRESORPTIVE AGENTS

a) Bisphosphonates

Bisphosphonates have emerged as the most effective drugs currently approved for the prevention and treatment of osteoporosis. They bind strongly to bone mineral especially at sites of active bone remodeling and are internalized by osteoclasts to inhibit resorption.

Functionally, they are the synthetic analogues of naturally occurring inorganic pyrophosphate, replacing P-O-P bond with P-C-P bond, which is highly resistant to hydrolysis. These can be further classified as nitrogen-containing (amino) bisphosphonates (alendronate, risedronate, ibandronate, pamidronate and zoledronate), which inhibit the
farnesyl pyrophosphate (FPP) synthase, an enzyme in the mevalonate pathway, and prevent prenylation of small guanosine triphosphate (GTP)-binding proteins (GTPases), which are essential for osteoclast function and survival. Secondly, non-nitrogen containing bisphosphonates (etidronate, tiludronate and clodronate) that are metabolized to cytotoxic ATP analogues, which induce osteoclast apoptosis (Das & Crockett, 2013). These drugs can be administered both orally and intravenously.

The major drawback of all bisphosphonates is that the absorption rate is less than 1% orally and long projected time before or after any food or other medication. Further, only 50% of the absorbed dose binds to bone and 50% is excreted by the kidneys. However oesophageal irritation, abdominal pain, nausea, dyspepsia, and gastrointestinal ulcers are common side effects (Das & Crockett, 2013). Current data indicates that alendronate, risedronate, ibandronate and zoledronate reduce the risk of vertebral fractures, as well non-vertebral and hip fractures in postmenopausal osteoporotic women by 40-70% over a 3-year period of time compared with placebo.

b) Selective estrogen receptor modulators (SERMs)

SERM are the synthetic compound that acts as estrogen agonists on bone and estrogen antagonists on breast and brain tissue. Raloxifene is the only approved second generation SERM by FDA for the prevention and treatment of osteoporosis in postmenopausal women. It acts as an estrogen receptor modulator which competes with estrogens for binding to the estrogen receptor. It functions as estrogen agonist at bone and liver by maintaining BMD and lowers LDL cholesterol levels as well an estrogen antagonist in breast and endometrial tissue. Women having contradictions with bisphosphonates due to side effects such as gastrointestinal intolerance, acid reflux and dosing requirements may have these as alternate therapy. The results based on the STAR (Study of Tamoxifen And Raloxifene) trial, raloxifene has been approved by the U.S Food and Drug Administration for risk reduction of breast cancer in postmenopausal women at increased risk (Provinciali, Suen, Dunn, & DeCensi, 2016). Bazadoxifene and Lasofoxifene are the 3rd generation SERMs that are approved by the European Union for the treatment of osteoporosis in postmenopausal women at increased risk of fracture (Gallaghera & Tella, 2013). However, these drugs may also be associated to cause venous thromboembolism or unexplained uterine bleeding and hot flushes.
c) **Calcitonin**

Calcitonin is a peptide hormone produced by the C-cells of the thyroid gland, has an antiresorptive effect on osteoclasts expressing calcitonin receptors. Salmon calcitonin is 20-40 times more potent than human calcitonin in decreasing bone loss and increasing bone density. Nasal calcitonin can be administered intranasally at a dose of 200 units per day (to alternate nostrils) and is generally well tolerated. Although, the side effects of nasal spray are rhinitis, headache, mild epistaxis and flushing (Daroszewska, 2012).

d) **Denosumab**

Denosumab is a fully humanised monoclonal antibody that specifically binds to Receptor Activator of Nuclear factor Kappa B Ligand and prevents RANKL from binding to RANK, thereby inhibiting osteoclastogenesis and osteoclast survival. The U.S FDA has approved denosumab 60mg, given subcutaneously at 6-monthly intervals for the treatment of osteoporosis in postmenopausal women and men, and bone loss associated with hormone ablation in men with prostate cancer at increased risk of fractures (Langdahl et al., 2015).

e) **Calcium and vitamin D**

Low calcium intake for prolonged period cause a negative calcium balance with a compensatory increase in PTH-mediated bone resorption. As a result, low bone mass is attained at younger age and further, later increases age-related bone loss and in postmenopausal women contributing to osteoporosis. The recommended daily calcium allowance according to the US Institute of Medicine (IOM) is 1300mg for adolescents, 1000mg for women till 50 age with tolerable upper level (UL) value of 3000mg and 2500mg respectively. These values may vary for postmenopausal women to 1200mg and 2000mg daily calcium allowance and the UL respectively (Daroszewska, 2012). Vitamin D is endogenously synthesized in the skin on exposure to the UVB light, but many factors, such as latitude, overcast sky, skin pigmentation and ageing, clothing and the use of sun blocks diminish this process. According to the IOM, the recommended daily allowance of Vitamin D are 600 International Units (IU) increasing to 800 IU after the age of 70 with the UL of 4000 IU assuming minimal sun exposure. Vitamin D deficiency leads to osteoporosis, osteopenia and osteomalacia, along with weakening of the muscles, autoimmune, cardiovascular, neoplastic, mental and infectious diseases. Vitamin D is widely available as ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3), which can be metabolized to
active metabolites to promote the active absorption of calcium and phosphorus by the small intestine, thereby elevating serum calcium and phosphate levels sufficiently to permit bone mineralization (Singh, Goyal, & Singh, 2013).

**f) Hormone replacement therapy**

Accelerated bone loss in women due to estrogen deficiency at menopause is characterized by relative osteoclasts activity greater than osteoblasts, resulting in net bone loss. Thus suppression of osteoclast activity by estrogen replacement (hormone replacement therapy, HRT) has been effectively used for decades and was the only source of prevention and treatment of osteoporosis in postmenopausal women before bisphosphonates. According to Women’s Health Initiative, estrogen with or without progesterone, slightly reduced the risk of hip and vertebral fractures, but in long term treatment associated with increased risk of stroke, venous thromboembolism, myocardial infarction, and breast cancer, even for women at high risk of fractures. Additionally, U.S FDA recommends hormone therapy only in women with high fracture risk, with moderate or severe vasomotor symptoms, using the lowest effective dose for the shortest time (Sweet, Sweet, Jeremiah, & Galazka, 2009).

**2.6.2 ANABOLIC AGENTS**

**a) Teriparatide**

Parathyroid hormone (1–84; PTH), is the important hormonal regulator of calcium homeostasis, has dual effect on bone, by indirectly (via osteoblasts) stimulating osteoclasts to resorb bone and bone formation at intermittent dose. Teriparatide (1–34 amino acid peptide) is a recombinant human PTH analog with potent osteoanabolic effect when administered intermittently at low doses. Teriparatide (Forteo®) is approved by the U.S FDA as an anabolic treatment for osteoporosis in individuals at high risk of fracture. Its daily administration, subcutaneously at effective dose of 20μg/day has shown increase BMD in postmenopausal (Finkelstein, Wyland, Lee, & Neer, 2010) and glucocorticoid-induced osteoporosis and has better effect than alendronate in reducing the incidence of vertebral and hip fractures (Saag et al., 2007). A combination of anti-resorptive therapy (to inhibit bone loss) with teriparatide therapy (to stimulate bone formation) has agent-specific effects on the overall effectiveness of teriparatide therapy.
b) *Strontium ranelate*

Strontium ranelate is novel therapy developed in the treatment of osteoporosis. It consists of two strontium atoms attached to ranelic acid moiety. It has dual effect by stimulating bone formation and decreasing bone resorption; as shown *in vitro* it enhances osteoblastic activity, by upregulating collagen synthesis and modulating the OPG/RANKL system favoring OPG synthesis. It also inhibits bone resorption by downregulation of osteoclast differentiation and resorption activity and leading to increase osteoclast apoptosis. Strontium ranelate is not FDA approved, but is licensed (oral formulation of 2g/day) in selected countries of Europe for restricted use for the prevention of vertebral and non vertebral osteoporotic fractures, in patients where bisphosphonate treatment has failed or is contraindicated. It is also associated with adverse effects of treatment, including skin rashes and deep vein thrombosis (Rene Rizzoli & Reginster, 2011).

### 2.7 OXIDATIVE STRESS AND OSTEOPOROSIS

Oxidative stress is characterized by the increased level of reactive oxygen species (ROS) that disrupts the intracellular reduction–oxidation (redox) balance causing a chain of cellular damage including lipids, membranes, proteins and nucleic acids (Wauquier, Leotoing, Coxam, Guicheux, & Wittrant, 2009). Epidemiological evidence in humans and animal studies have suggest that aging and high ROS are associated in the pathogenesis of various chronic diseases including cancer, atherosclerosis, diabetes, neurodegenerative disorders, arthritis and osteoporosis. Furthermore, enhanced generation of intracellular ROS such as superoxide anions, hydrogen peroxide and imbalance in intrinsic antioxidant defense systems, cause oxidation of lipids, DNA, and proteins, leading to bone loss in osteoporosis by free radical oxidative stress.

Although under physiological conditions, activated osteoclasts generate ROS which accelerates the destruction of calcified tissue by releasing a nicotinamide adenine dinucleotide phosphate-reduced (NADPH) oxidase enzyme that is capable of cytokine-regulated ROS generation. Due to estrogen deficiency, ROS induces TNF-α, and other cytokines production in many cells leading to bone loss (Sheweita, Khoshhal, & Baghdadi, 2014). The endogenous antioxidant enzymes include catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) and metal chelating proteins. Dietary sources of
exogenous antioxidants includes lycopene, a lipid soluble carotenoid, flavonoids, water soluble polyphenols, Vitamin C and E (Rao & Rao, 2013).

2.8 MATERNAL NUTRITION AND FUTURE OSTEOPOROSIS RISK

The availability of nutrients in utero may directly influence the foetal development at critical points, acting on biologically plastic processes during development, with potential long-term health outcomes (Barker, 2012; Christian & Stewart, 2010). During pregnancy, diet has shown to be associated with epigenetic changes altering postnatal transcriptional activity of genes that affect childhood body composition (Godfrey et al., 2011) and possibly bone mass. Several cohort studies imparts useful information on how maternal behavior influences later bone health. The association of future risk has been observed first in one of the cohort study in young women born in Bath (E M Dennison et al., 2010).

According to Hertfordshire cohort study, poor growth in utero and early stage of postnatal life is associated with alterations in bone architecture, cortical size and geometry, reduction in bone mineral content, resulting in compromised bone strength and increased risk of later fracture (Elaine M. Dennison, Syddall, Sayer, Gilbody, & Cooper, 2005). In a Southampton Women’s Survey, it was shown that mRNA expression of active plasma membrane calcium transporter (PMCA3) is positively correlated to the newborn bone mass, concluding that maternal vitamin D insufficiency might alter placental calcium transport. Moreover, vitamin D and calcium supplementation could lead to long-lasting reductions in the risk of osteoporotic fracture in the offspring (Javaid et al., 2006).

In cohort study from India, it has been shown that children born to mothers who were dependent on higher uptake of calcium-rich foods during pregnancy had higher total and spine bone mineral content and BMD independent of parental size and DXA measurements (Ganpule et al., 2006). Offspring of pregnant rats fed with low-protein subsequently develop functional impairment in adulthood, including hypertension, progressive deterioration of renal function, cardiovascular disease, impaired immune response and altered lifespans (Stuart A Lanham, Roach, Cooper, & Oreffo, 2010).

2.9 IN VIVO ANIMAL MODELS FOR OSTEOPOROSIS

According to the WHO guidelines, the primary objective of preclinical testing is to evaluate pharmacodynamic effects, particularly on bone mass and bone density measurement by
biomechanical testing, microarchitecture and histomorphometric variables and biochemical indices of skeleton turnover, which is the direct measure of osteoporosis. The animal models should have following characteristics (World Health Organization, 1998).

- Increased bone turnover after ovariectomy.
- Bone loss leading to an osteoporotic state that is not spontaneously reversible.
- Bone loss affecting both cortical and cancellous tissue at relevant skeletal sites such as vertebral body, femoral neck, metaphysis and diaphysis of long bones.
- Increased skeletal fragility.

Animal models used for antiosteoporotic studies include dogs, cats, non-human primates, ferrets, sheep, rodents, rabbits, guinea pigs and minipigs (S. A. Turner, 2001). In mice, a high bone loss of cancellous region but not cortical bone is seen soon after ovariectomy, that is recovered by 17-β-estradiol treatment in ovariectomized mice. Ovariectomized rat model is commonly preferred animal model for osteoporosis, as it exhibits majority features of human postmenopausal osteoporosis. For new pharmacological drug efficacy, toxicity and preliminary screenings, OVX rats are preferred as starting points and also have advantage viz numerous, inexpensive, easy to house and handle (Aerssens, Boonen, Lowet, & Dequeker, 1998).

2.10 GLUCOCORTICOID-INDUCED OSTEOPOROSIS

Glucocorticoids are class of steroids hormones, that have been widely used to treat inflammatory, allergic disorders and immunosuppressive agents that cause asthma, chronic lung disease, rheumatoid arthritis and other connective tissue diseases, inflammatory bowel disease, and after organ transplants. Endogenous overproduction or systemic administration of glucocorticoids is the most common cause of secondary osteoporosis seen in patients of all ages, from children to the elderly and common iatrogenic cause of osteoporosis. Deleterious effect of prolonged exposure to high levels of glucocorticoids was first recognized in Cushing’s syndrome, resulting in muscle wasting and weakness, skin thinning Osteoporosis and bone fracture, and fat redistribution from the periphery to the center of the body and growth arrest in children (Fernandez-Rodriguez, Stewart, & Cooper, 2009).
2.10.1 PATHOPHYSIOLOGY

Glucocorticoid Induced Osteoporosis (GIOP) has both direct and indirect effects on bone tissue that leads to bone loss and reduced bone formation, which may probably constitutes the main difference between GIOP and postmenopausal osteoporosis that characterized by increased bone turnover (Weinstein, 2011). The direct effect has shown that glucocorticoids (GC) rapidly suppresses several indices of osteoblast activity, including serum propeptide of type I N-terminal procollagen (P1NP), propeptide of type I C-terminal procollagen (P1CP) and osteocalcin even at low doses (Ton, Gunawardene, Lee, & Neer, 2005). Bone resorption increases with initial exposure to glucocorticoids leading to more chronic and progressive bone loss with rapid decline in BMD, impaired bone formation and fracture risk.

Glucocorticoid promotes osteoclastogenesis, by reduction in osteoclast apoptosis through both increased expression of macrophage stimulating factor (MCSF), receptor activator of nuclear factor κB ligand (RANKL) and inhibition of osteoprotegerin (OPG) a soluble decoy receptor of RANKL, in stromal and osteoblastic cells (L. C. Hofbauer et al., 1999; Hyun-ju Kim et al., 2006). Further, it involves inhibition of Wnt/beta-catenin signalling pathway and runt-related protein 2 (RUNX2); upregulation of Dickkopf-related protein 1 (DKK-1) and sclerostin negative regulators of bone formation, expression of peroxisome proliferator-activated receptor γ2 (PPARγ2) (Yao et al., 2008).

Glucocorticoids also accelerate the differentiation of bone marrow stromal cells towards adipogenesis instead of osteoblastogenesis (Uyl, Bultink, & Lems, 2011). In the human studies, early exposure to glucocorticoids has a rapid loss in bone mineral density (BMD) and excessive bone resorption, whereas slow late phase leads to impaired bone formation. Glucocorticoids uptake reduced the calcium absorption from the gastrointestinal tract by mechanisms that oppose vitamin D action and also decrease the renal tubular calcium reabsorption. Consequently, deleterious effects of these actions lead to the development of secondary hyperparathyroidism (Ernesto Canalis, Bilezikian, Angeli, & Giustina, 2004). Indirectly, GC also suppresses the expression of insulin-like growth factor 1 (IGF-1) by skeletal cells and reduced secretion of growth hormone (GH), that may further interfere the GH/IGF-1 axis. Moreover, it obstructs the release of gonadotropins, which results in reduced estrogen and testosterone production and causing hypogonadism and bone loss (Mirza & Canalis, 2015). Currently, bisphosphonates and teriparatide have been assessed in prevention and treatment of GIOP, but still associated with efficacy issues.
Nowadays, it is estimated that approximately 80% of the world population primarily in developing countries still rely on traditional medicine based largely on plants and animals for their primary health care. The increased popularity and demand of herbal medicines is due to their effectiveness, fewer side effects and relatively low cost compared to their counter synthetic drugs related to adverse side-effects or lack of efficacy. This has stimulated the interest of scientists and doctors towards traditional medicines for treatment of some chronic diseases, including osteoporosis (Verma & Singh, 2008). India being the vast repository of medicinal plants around 20,000 medicinal plant species has been recorded and about 800 plant species are used for curing different diseases. Currently, plant-derived medicines form the first line of primary health care for human alleviations for the 80% of the world population and also the important sources of medicines. Presently, in the United States about 25% of pharmaceutical prescriptions contain at least one plant-derived ingredient. It has been estimated that the market of Ayurvedic medicines is expanding at 20% annually with expected export of Rs. 1200 million annually from India, with constant growth of 15% in all major herbal-based pharmaceutical companies.
Phytoestrogens (PE) are polyphenolic, non-steroidal plant-derived compounds that share structural and functional similarities with mammalian estrogen, estradiol, and bind to estrogen receptors (ERs) preferably with ERβ. They provide potential alternatives to the synthetic selective estrogen receptor modulators (SERMs) and Hormone replacement therapy by alleviating menopausal symptoms with beneficial effects on cardiovascular diseases, prostate cancer and breast cancer, by improving the defensive system. The main group of phytoestrogens consist of isoflavonoids, flavonoids (flavones, flavanones, chalcones), coumestans, lignans and stilbenes (Resveratrol) (Pankova & Tsvetkova, 2015). The main food sources rich in phytoestrogens are nuts, oilseeds, soy products, cereals, breads, legumes, meat products and processed foods that may contain soy, vegetables, fruits, alcoholic and nonalcoholic beverages. Flax seed and other oilseeds contained the highest total phytoestrogen content.

Further studies have shown positive effects of several plant extracts on bone turnover, bone mineral density (BMD) and bone health. Flavonoids such as icariin, epimedim B, and epimedin C have found to inhibit bone resorption, stimulate bone formation, suppress urinary calcium excretion without uterus hyperplasia in the ovariectomized rats, indicating their antiosteoporotic potential. Further, Icariin exerts anabolic effect on bone by increasing estrogen receptor (ER) dependent cell proliferation, ALP activity, OPG/RANKL ratio in UMR 106 cells, increased ERα phosphorylation, decreased TRAP activity of osteoclasts, inhibits LPS-induced bone resorption and decreased expression of IL-6 and TNF-α (Jia et al., 2012). *Trifolium pratense* L. (red clover) is rich source of daidzein, genistein, formononetin and biochanin A isoflavones, are effective in reducing bone loss induced by ovariectomy.

Daidzein inhibits the osteoclast proliferation and differentiation by increasing apoptosis of osteoclast progenitors mediated by ERs (Kawakita et al., 2009). Kaempferol exerts protective effect on antimycin A-induced cell damage by mitochondrial membrane potential dissipation, complex IV inactivation, ROS production through activation of PI3K (phosphoinositide 3-kinase), Akt (protein kinase B), CREB (cAMP-response element-binding protein) in MC3T3-E1 (E. M. Choi, 2011). *Cissus quadrangularis* (CQ), *Glycine max* (soy) and *Linum usitatissimum* (flaxseeds) are rich source of phytoestrogens which are utilized for preventing and treating menopausal related disorders and have been proven by non clinical (Di Pompo et al., 2014) and clinical studies (Brahmkshatriya et al., 2015; Lemay et al., 2002; Messina, 2014). Various medicinal plants have been documented for the antiosteoporotic
activity related to their chemical constituents, mechanism of action and application in the prevention and treatment of osteoporosis (Shirwaikar, Khan, Kamariya, Patel, & Gajera, 2010) (Table 2).

Table 2 List of Antiosteoporotic medicinal plants.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Common Name</th>
<th>Biological Source</th>
<th>Chemical Constituents</th>
<th>Proposed Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hadjod</td>
<td>Cissus quadrangularis</td>
<td>Steroids, alkaloids, calcium</td>
<td>Rich in calcium. Osteoclastic inhibition</td>
</tr>
<tr>
<td>2</td>
<td>Black Cohosh</td>
<td>Cimicifuga racemosa</td>
<td>Flavonoids, triterpene glycoside and aromatic acids</td>
<td>Osteoclastic inhibition by ER binding to and aromatic acids</td>
</tr>
<tr>
<td>3</td>
<td>Soybean</td>
<td>Glycine max</td>
<td>Isoflavonoids; genistein daidzein, etc</td>
<td>Binding with estrogen receptor</td>
</tr>
<tr>
<td>4</td>
<td>Maca</td>
<td>Lepidium meyenii</td>
<td>Alkaloids, steroids, glucosinolates, macamides</td>
<td>By osteoclastic inhibition</td>
</tr>
<tr>
<td>5</td>
<td>Pila Bhangara</td>
<td>Wedelia calendulaceae</td>
<td>Isoflavonoids, wedelolactone</td>
<td>Not clear may act like SERM</td>
</tr>
<tr>
<td>6</td>
<td>Foetid bugbane</td>
<td>Cimicifuga foetida</td>
<td>Cimicifoetisides A and B, triterpenoids</td>
<td>Acts like SERMs</td>
</tr>
<tr>
<td>7</td>
<td>Red Clover</td>
<td>Trifolium pratense</td>
<td>Isoflavonoids like biochanin A and genistein</td>
<td>Estrogen Receptor binding</td>
</tr>
<tr>
<td>8</td>
<td>Japanese pagoda tree</td>
<td>Sophora japonica</td>
<td>isoflavonoids</td>
<td>Genistein like action</td>
</tr>
<tr>
<td>9</td>
<td>Herba epimeddi</td>
<td>Epimedium brevicornium</td>
<td>Icarin, flavonoids, sterols, fatty acids</td>
<td>Exact mechanism unknown.</td>
</tr>
<tr>
<td>10</td>
<td>Mushroom</td>
<td>Pleurotus eryngii</td>
<td>Polysaccharides, volvatoxin, ganoderic acid</td>
<td>Anti bone resorption and bone forming</td>
</tr>
<tr>
<td>11</td>
<td>Fructus Ligustri Lucidi</td>
<td>Ligustrum lucidum</td>
<td>Oleanolic acid, lupeol, betulin, fatty acids</td>
<td>Direct stimulation of osteoblasts</td>
</tr>
<tr>
<td>12</td>
<td>Tea</td>
<td>Camellia sinensis</td>
<td>Polyphenols and flavonoids</td>
<td>Antiresorptive due to estrogenic</td>
</tr>
<tr>
<td>13</td>
<td>Chinese Foxglove</td>
<td>Rehmannia glutinosa</td>
<td>Steroids, norcarotenoids, remophilanetriol</td>
<td>Anti bone resorption and bone forming</td>
</tr>
</tbody>
</table>
14 Jie gu mu  
*Sambucus williamsii*  
(Caprifoliaceae)  
Steroids, triterpenoids, phenolic acid  
By decreasing osteoclastogenesis

15 Onobrychis  
*Onobrychis ebenoides*  
(Leguminosae)  
Arylobenzofurans and isoflavonoid  
Binding to ERα-ERE and ERβ-ERE

16 Indian coral tree  
*Erythrina variegate*  
(Leguminosae)  
Isoflavonoids, Sphaerobioside, Orientanol B  
Phytoestrogens like actions

17 Safflower  
*Carthamus tinctorius L.*  
(Asteraceae)  
Flavonoids, Kinobean A, fixed oil  
Osteoblastic stimulation

18 Bawachi  
*Psoralea corylifolia*  
(Fabaceae)  
Furano-coumarins, Flavonoids, terpenoids  
Osteoblastic stimulation

Adapted from: Medicinal Plants for the Management of Post Menopausal Osteoporosis: A Review

### 2.12 Selection of *Sesbania grandiflora* (SG) plant for Antiosteoprototic activity

*Sesbania grandiflora* (L.) Pers. (Leguminosae), is an Indian medicinal plant, also known as ‘sesbania’, ‘agathi’, ‘humming bird tree’ and listed Ayurvedic drug in Indian Materia Medica (Nadkarni, 1982).

#### 2.12.1 Ethanomedicinal Uses

All parts of *Sesbania grandiflora* are utilized in the treatment of various disorders as: bruises, catarrh, dysentery, eyes, fever, headache, smallpox, sores, sore throat, anemia, bronchitis, nasal catarrh, inflammation, leprosy, gout and rheumatism, stomatitis, antiulcer, swellings and tumors. Classically, it is used in gynecological applications for leucorrhoea, amenorrhea, anemia, emaciation, milk stimulation after child birth (Loganayaki, Suganya, & Manian, 2012; Nadkarni, 1982) and gonorrhea in males.

#### 2.12.2 Phytochemical Aspects

*S. grandiflora* possess rich source of phytoestrogens including flavonoids (catechin, epicatechin, luteolin, kaemferol-3-rutinoside, myricetin, naringenin and quercetin,) and other phyto-compounds such as beta-carotene, cyanidin, glycosides, grandiflorol, leucocyanidin, neoxanthin, oleanolic acid, sesbanimide, tannin, violaxanthin, vitamins, zeaxanthin and
saponins which on hydrolysis gave an acid sapogenin (Mustafa et al., 2010; Wagh, Wagh, Tandale, & Salve, 2009).

2.12.3 Pharmacological studies

a) Antioxidant, anti-inflammatory and analgesic activity

Various extracts of *S. grandiflora* have been shown to exhibit antioxidant, anti-inflammatory, and analgesic activities. Pharmacological studies reviewed the antioxidant, anti-inflammatory and antinociceptive activity of methanolic extract of *S. grandiflora* flowers. The extract exhibited potent cytotoxic potential against human cervical cancer cell line HeLa and significant inhibitory activity against inflammation in carrageenan, hot plate and cotton pellet induced models, which may be due to the presence of flavonoids and phenolic compounds (Loganayaki et al., 2012). Swetha et al in 2012 have reported that 500mg/kg dose of ethanolic extract of stem bark of *Sesbania grandiflora* have shown significant analgesic and antiinflammatory activity *in vivo* when compared with the standard drug diclofenac sodium (Swetha et al., 2012).

b) Cardioprotective and Neuroprotective

Ramesh et al 2008, have shown that treatment with aqueous suspension of *S. grandiflora* leaves have cardioprotective effect in cigarette smoke–exposed rats with improvement in defense mechanisms. The results depicted enhanced levels of cardiac superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, glucose-6-phosphate dehydrogenase and restoration of micronutrients (Ramesh, Mahesh, & Sureka, 2008). The same group also reported the neuroprotective effect of *S. grandiflora* leaves on brain oxidative damage in cigarette smoke-exposed rats by stabilizing their cell membranes through restoration of protein carbonyl, nitric oxide, ROS-producing endogenous enzymes, nucleic acids, thiols, lipids, membrane bound ATPases and maintenance of ion homeostasis (Ramesh, Sureka, Bhuvana, & Begum, 2015a, 2015b).

c) Antidiabetic activity

Nandi et al 2014, reported the antidiabetic activity of methanolic extract of *S. grandiflora* fruit. The extract at both the doses 200mg/kg and 400mg/kg attenuate the plasma glucose levels in Type-2 diabetes mellitus models in streptozotocin induced diabetic rats. Further, it
improved the aberrant lipid profile, oxidative stress and insulin sensitivity suggesting its anti-
hyperglycemic potential (Nandi, Garabadu, Krishnamurthy, Trayambak, & Singh, 2014).
Various reports have demonstrated the antihyperglycemic potential of S. grandiflora leaves in
different experimental model of alloxan or streptozotocin induced diabetic rats at varying
dose concentrations. The results exhibited restoration of plasma glucose, serum insulin,
glucosylated hemoglobin, hepatic glycogen, glucokinase, glucose-6-phosphatase and serum
marker enzymes (Ghanshyam et al., 2012; Panigrahi, Panda, & Patra, 2016; Radhika, Ruth
Christia, & Jothi, 2014; Sangeetha, Prasath sriram, & Subramanian, 2014).

d) Anticancer and Antiproliferative Activity

It was stated that the ethanol extract of S. grandiflora flower showed anticancer activity with
significant decrease in tumor volume, cell viability, tumor weight and elevated life span of
Ehrlich Ascites Carcinoma bearing mice comparable to 5-Fluorouracil (Sreelatha, Padma, &
Umasankari, 2011). The methanolic extract of S. grandiflora exerts potent antiproliferative
and apoptotic effects on human cancer cells especially on human lung cancer cell line, A549.
The mechanistic action of cancer cell death may be via G1/S arrest through decreased cyclin
D1 levels and prevention of NFκB pathways, which triggers the apoptosis (Pajaniradje,
Mohankumar, Pamidimukkala, Subramanian, & Rajagopalan, 2014). Further reports stated
that fraction from S. grandiflora flowers have apoptotic and autophagic effects on human
leukemic cells particularly of histiocyte lymphoma via programmed cell death (Roy, Kumar,
Chakraborty, Chowdhury, & Das, 2013).

e) Hepatoprotective Activity

Kale et al 2012, demonstrated the Hepatoprotective potential of ethanolic and aqueous extract
of flowers of Sesbania grandiflora (Linn) induced by CCl4, probably by stimulating hepatic
regeneration via improved protein synthesis or release of microsomal activation to toxicants
(Kale, Khan, Irfan, & Veerana, 2012). Tathe et al 2010 also summarized the hepatoprotective
activity of Sesbania Grandiflora fruit extract in alcohol induced-hepatotoxicity model in rats
(Tathe et al., 2010).

f) Anxiolytic and anticonvulsive Activity

Chopde et al 2002 demonstrated the anxiolytic and anticonvulsive activity of benzene:ethyl
acetate fraction (BE) of S. grandiflora leaves in pentylenetetrazol (PTZ) and strychnine
(STR)-induced seizures in mice. The BE fraction majorly contained triterpenes. As observed, mice treated with BE preferred to stay in the open arm of the elevated plus maze test indicating anxiolytic activity that may be due an increase in brain contents of gamma-aminobutyric acid and serotonin (Kasture et al., 2002).

g) Antiurolithiatic and Wound healing activity

The leaf juice of *S. grandiflora* exhibited significant antiurolithiatic activity against calcium oxalate-type stones with no gross behavioral changes except for an increase in urination (Doddola, Pasupulati, Koganti, & Prasad, 2008). The methanolic extract of bark of *Sesbania grandiflora* (L.) was evaluated for wound healing activity using excision wound model in Wistar albino rats. The extract showed significant healing at 10% w/w dose comparable to the standard 1% framycetin sulphate that may be due to the synergistic effect of both inhibition of lipid peroxidation and antimicrobial activity (Karthikeyan, Suresh, & Suresh, 2011).

The present study systematically researched the effects of crude ethanol and aqueous leaf extracts of *S. grandiflora* (SG) in osteoporotic rat model and the toxicity after repeated oral administration. We hypothesized that *S. grandiflora* may beneficially prevent bone loss caused by estrogen deficiency. Our data suggest that *S. grandiflora* treatment is safe, inhibits bone deterioration in OVX and glucocorticoid rat model likely via new bone formation, suggesting its role as a protective agent for mediating bone diseases.