Introduction
**BRIEF BACKGROUND:**

Osteoporosis is a heterogenous metabolic disorder characterized by a progressive loss of bone tissue in the increasingly ageing population responsible for the increase in bone fragility with a tendency to fracture following minimal trauma. Osteoporosis currently affects up to 1 in 3 women and 1 in 12 men at some point in their lives (Ligett & Reid, 2000). Approximately 1.6 million hip fractures occur worldwide, the incidence is set to increase to 6.3 million by 2050. In India, 1 out of 8 males and 1 out of 3 females suffers from osteoporosis, making India one of the largest affected countries in the world. The number of osteoporotic patients is pegged at approximately 26 million (2003 Figures) with the numbers projects to increase to 36 million by 2013 (Sophie et al., 2007).

**CURRENT TREATMENT OF OSTEOPOROSIS AND ITS DISADVANTAGES:**

Pharmacological treatments available for osteoporosis are divided into two broad categories:

1. **Antiresorptive**, which include bisphosphonates, HRT, Selective estrogen receptor modulators (SERMs), calcitonin, vitamin D analogues and combination therapy of calcium and vitamin D.

2. **Anabolic**, which include sodium fluoride, anabolic steroids and PTH (Teriparatide).

Although currently in use, all have serious disadvantages, for example, the bisphosphonates, the most widely used drugs for the treatment of established osteoporosis, act by binding to hydroxyapatite and inhibiting osteoclasts at their resorption sites and must be taken on an empty stomach. Side effects include nausea, vomiting, diarrhoea, peptic ulceration, abdominal pain and constipation along with allergic skin reactions and the possibility of osteomalacia (Ferguson, 2004). HRT confers an increased risk of pulmonary embolism and breast cancer, and has many contraindications (Ferguson, 2004). There is insufficient evidence of benefits, but reliable evidence of harm (Bruyere et al., 2003). SERM, for example, raloxifene, acts in a similar manner to HRT but with less of a risk of breast cancer, however, still carries the risk of venous thromboembolism (Ferguson, 2004).
Calcitonin, polypeptide hormone produced by the thyroid gland, acts to decrease bone resorption by inhibiting the activity of osteoclasts through binding to calcitonin receptors on the osteoclast. Side effects include flushing, nausea, vomiting, and diarrhoea. Calcitriol (1, 25-dihydroxy vitamin D3) increases intestinal absorption of calcium and promotes mineralization of bone. Its efficacy is mainly proven in milder cases of osteoporosis whilst side effects include hypercalcaemia and hypercalciuria. Anabolic agents increase the number and activity of osteoblasts, but also of osteoclasts, resulting in a net increase of bone at cortical sites. Side effects of the drug are male characteristics in women, and liver toxicity (Ferguson, 2004).

PTH acts primarily to sustain or increase plasma calcium levels. PTH has anabolic effects on bone, which result from binding to receptors on osteoblast surface, thus promoting osteoblast cell proliferation via a signaling pathway involving two second messengers (Berne & Levy, 1998). PTH and PTH-related protein (PTHrP), low doses of both are the most promising anabolic treatment for osteoporosis thus far however, high levels can cause increased bone resorption and osteitis fibrosa cystica (Manologas, 2000). Future studies to assess the antifracture efficacy of these compounds will be needed before their effectiveness for the management of osteoporosis can be established.

**BONE PATHOPHYSIOLOGY:**

Bone is a complex tissue composed of cells, collagenous matrix, and inorganic elements. It provides many essential functions, including mechanical support, protection of vital organs, a microenvironment for hematopoiesis, and a depot for calcium and other minerals. The growth, development, and maintenance of bone are highly regulated process (Nijweide et al., 1986). The level of bone mass reflects the balance of bone formation and resorption, which at the cellular level involves the synthesis of bone matrix by osteoblasts and the coordinate resorption of bone by osteoclasts (Felix et al., 1996; Roodman, 1996). Osteoblasts and osteoclasts arise from distinct cell lineages and maturation processes; - osteoblasts arise from mesenchymal stem cells, whereas osteoclasts differentiate from hematopoietic monocytes/macrophage precursors. Both cell types are influenced by a wide variety of hormones, inflammatory mediators, and growth factors (Suda et al., 1992; Mundy,
An imbalance of osteoblast and/or osteoclast functions can result in skeletal abnormalities characterized by increased (osteopetrosis) or decreased (osteoporosis) bone mass.

Low bone mass results due to insufficient bone deposited in the skeleton during growth or due to subsequent loss of bone tissue at an excessive rate. Several forms of osteoporosis have been identified. Osteoporosis can be therefore, classified as primary which includes postmenopausal (Type I) or secondary which includes senile (Type II) osteoporosis. The Type I variety occurs typically between 55-75 years, affects mainly trabecular bone and is more common in women than in men (6:1 ratio). Prior to menopause, bone loss occurs at the rate of 0.5-1% per year. At menopause, bone loss accelerates at the rate of 2-5% per year due to decline in estrogen levels and is greatest in the first 3-6 years post menopause. The Type II or age-related osteoporosis occurs after the age of 70 years, affects both cortical and trabecular bone inflicting women twice as frequently as men. Secondary osteoporosis can occur due to specific causes such as an endocrine disease (e.g., hyperparathyroidism, hyperthyroidism, glucocorticoid excess, hypogonadism, etc.), drug induced (e.g., glucocorticosteroids, barbiturates, heparin, alcohol, etc.), congenital conditions (e.g., homocystinuria, hemolytic anemia, hypophosphatasia, osteogenesis imperfecta), diet conditions (e.g., anorexia nervosa, calcium deficiency, malabsorption syndromes, etc.) and miscellaneous conditions (e.g., prolonged immobilization, rheumatoid arthritis, chronic liver failure, diabetes mellitus, etc.).

TNF FAMILY MOLECULES IN PHYSIOLOGICAL AND PATHOLOGICAL BONE RESORPTION

a) RANKL:

Human RANKL [also called TNF-related activation-induced cytokine (TRANCE)/ osteoprotegerin ligand (OPGL) /osteoclast differentiation factor (ODF)], a cytokine independently discovered by four different groups (Wong et al., 1997; Anderson et al., 1997; Yasuda et al.,1998; Lacey et al., 1998), is a member of the TNF ligand superfamily. It is a cytokine family that also includes TNF- α, TNF- α CD40 ligand, Fas ligand, CD30 ligand, TWEAK, and TRAIL.
Introduction

(Locksley et al., 2001). Like other members of the TNF-like family of cytokines, RANKL is a type II membrane-embedded protein, with a large extracellular receptor-binding domain, a membrane-anchoring domain, and a connecting stalk. The RANKL gene is present on human chromosome 13q14 and on mouse chromosome 14. The mouse and human RANKL consists of 316 and 317 amino acid (aa) residues, respectively. The homology within the TNF ligand family is confined to domains involved in monomer folding and trimer assembly. The shape of the ligand is that of an inverted bell, which at the base interacts with the receptors in 3:3 symmetric complexes. The trimeric protein contains four unique surface loops that create the specificity in its interaction with the receptor RANK (Lemer, 2004). Like several other TNF-like type D proteins, RANKL trimers exist either as membrane-anchored proteins or in a soluble cleaved form, both being functionally active (Nakashimu et al., 2000).

RANKL mRNA is expressed at the highest levels in bone and bone marrow and in lymphoid tissues (lymph node, thymus, spleen, fetal liver, and Peyer’s patches) (Wong et al., 1997; Anderson et al., 1997; Yasuda et al., 1998; Lacey et al., 1998). Its major role in bone is the stimulation of OC differentiation (Lacey et al., 1998; Malyankar et al., 2000) and inhibition of OC apoptosis (Fuller et al., 1998). In addition, RANKL has a number of effects on immune cells, including activation of c-Jun N-terminal kinase (JNK) in T cells (Wong et al., 1997), inhibition of apoptosis of dendritic cells (Wong et al., 1997), and alterations in cytokine-activated T cell proliferation (Anderson et al., 1997). RANKL knockout mice have severe osteopetrosis (Kong et al., 1999) and complete absence of OCs. In addition, they exhibit defects in early differentiation of T and B cells, lack lymph nodes, and have defects in thymic differentiation and mammary gland development (Kong et al., 1999; Fata et al., 2000). The promoter region of the RANKL gene contains a response element for core-binding factor α1 (Cbfa-1 or Runx2) which is a transcription factor crucial for OB differentiation and expression of bone matrix proteins (Ducy et al., 1997)
b) **RANK:**

Receptor activator of nuclear factor kappa B (RANK), a member of the TNF receptor superfamily, is a type E transmembrane protein containing four cysteine-rich pseudorepeat domains in the extracellular region, a hallmark of the TNF-R family (Locksley et al., 2001). The mouse and human RANK contain 625 and 616 aa residues, respectively, the latter having a signal peptide (28 aa), an N-terminal extracellular domain (184 aa), a transmembrane spanning domain (21 aa), and a large C-terminal cytoplasmic tail (383 aa). In the human genome, RANK is present on chromosome 18q22.1. The extracellular part of TNF-R-like receptors forms elongated structures by a scaffold of disulfide bridges, which fit into the inverted bell/ groove of the ligand trimer in a 3:3 complex (Locksley et al., 2001). This has recently been demonstrated to be true for the interaction between RANKL and RANK (Lam et al., 2001). Although RANK is ubiquitously expressed in human tissues, its cell surface expression is limited to DC, the CD4+ T cell line MP-1, foreskin fibroblasts, OC precursors, and certain Hodgkin lymphomas (Anderson et al., 1997; Hsu et al., 1999; Fiumara et al., 2001). RANK knockout mice lack peripheral lymph nodes and have defective T and B cell maturation (Li J et al., 2000).

c) **OPG:**

Osteoprotegerin (OPG); also known as osteoclastogenesis inhibitory factor (OCIF) or TNF receptor-like molecule (TR 1), is a secreted protein that regulates bone mass by inhibiting OC differentiation and activation (Simonet et al., 1997; Yasuda et al., 1998; Tan et al., 1997). OPG is synthesized in humans, rats, and mice as a 401-aa protein, which, after cleavage of 21-aa signal peptide, results in a 380-aa mature protein. In humans, the OPG gene is located on chromosome 8q23-24. The aa sequence of OPG displays several homologies to members of the TNF-R superfamily, including RANK. The 60-kDa monomer OPG protein forms 120-kDa disulfide-linked homodimers containing several N-glycosylation sites. OPG contains four cysteine-rich domains in the N-terminal end, two homologous ‘death domains’ (DDH) in the C-terminus, a heparin-binding site, and a cysteine residue required for homodimerisation but, in contrast to other members of the TNF-R superfamily, lacks a transmembrane spanning domain.
Introduction

and a cytoplasmic tail. Secreted OPG acts as a ‘decoy receptor’ due to its affinity to both membrane-bound and soluble RANKL and prevents the activation of RANK. OPG mRNA has been detected in bone, cartilage, aorta, skin, lung, heart, kidney, liver, brain, and in several other tissues. At the cellular level, OPG is expressed in OBs, stromal cells, endothelial cells, aortic smooth-muscle cells, fibroblasts, dendritic cells, and lymphoid cell lines (Lerner, 2004).

Figure (a): The RANK/RANKL/OPG System

RANKL, expressed on the cell surface of osteoblasts/stromal cells, binds to RANK, expressed on the cell surface of hemopoietic osteoclast precursor cells, inducing a signaling and gene expression cascade that leads to their differentiation and maturation to osteoclasts. OPG, which can bind to RANKL, acts as a decoy receptor, blocking the interaction between osteoblasts/stromal cells and the osteoclast precursors, thereby inhibiting osteoclast formation. (Adapted from medscape.com)
CLINICAL UTILITY OF BIOCHEMICAL MARKERS OF BONE REMODELING:

Bone is a metabolically active tissue that undergoes continuous remodeling by two counteracting processes, namely bone formation and bone resorption. These processes rely on the activity of osteoclasts (resorption), osteoblasts (formation) and osteocytes (maintenance). Under normal conditions, bone resorption and formation are tightly coupled to each other, so that the amount of bone removed is always equal to the amount of newly formed bone [Figure (b)]. This balance is achieved and regulated through the action of various systemic hormones (e.g. PTH, vitamin D, and other steroid hormones) and local mediators (e.g. cytokines, growth factors). In contrast, somatic growth, ageing, metabolic bone diseases, states of increased or decreased mobility, therapeutic interventions and many other conditions are characterized by more or less pronounced imbalances in bone turnover.

Although the currently available markers of bone turnover include both enzymes and non-enzymatic peptides derived from cellular and non-cellular compartments of bone, they are usually classified according to the metabolic process they are considered to reflect. Most biochemical indices of bone resorption are related to collagen breakdown products such as hydroxyproline or the various collagen cross-links and telopeptides. Other markers of bone resorption include non-collagenous matrix proteins such as bone sialoprotein (BSP), or osteoclast-specific enzymes like tartrate-resistant acid phosphatase or cathepsin K. In contrast, markers of bone formation are either by-products of collagen neosynthesis (e.g. propeptides of type I collagen), or osteoblast-related proteins such as osteocalcin (OC) and alkaline phosphatase (AP). For clinical purposes, therefore, markers of bone formation are distinguished from indices of bone resorption [Figure (c)]. This distinction, however, is not as sharp as it may appear. For example, some marker components reflect, at least in part, both bone formation and bone resorption (e.g. hydroxyproline, certain OC fragments). Furthermore, most of the molecules used as markers of bone turnover are also present in tissues other than bone, and non-skeletal processes may therefore influence their circulating or urinary levels. Finally, changes in markers of bone turnover are not disease specific but reflect, as an integral measure, alterations in the metabolism of the entire skeletal envelope independently of the underlying cause.
Hence, results of bone marker measurements should always be interpreted against the background of their basic science and the clinical picture.

Figure (b): The bone remodelling cycle

Under normal conditions, the resorption (osteoclast) phase takes approximately 10 days, which is then followed by a formation (osteoblast) phase that can last for up to 3 months. (Adapted from Seibel MJ, 2005)
1. **BONE FORMING MARKERS:**

   a) **Alkaline phosphatase**

   Osteoblasts are rich in alkaline phosphatase; however, alkaline phosphatase, an enzyme associated with the plasma membrane of cells, is also found in liver, intestine, and placenta (Gomez B, Jr, Ardakani S, Ju J, et al., 1995), all of which may contribute to the total amount of alkaline phosphatase found in blood. The bone isoenzyme predominates in childhood and particularly during puberty; however, in adults the bone and liver isoenzymes contribute approximately equally to the total, with the intestinal fraction accounting for <10%. The function of alkaline phosphatase is unknown. The condition hypophosphatasia, in which the enzyme is lacking, is characterized by osteomalacia, suggesting that alkaline phosphatase has a role in the mineralization of newly formed bone. Measurement of total serum alkaline phosphatase is useful when the amount from bone is exceptionally high (such as in Paget disease of bone) and concentrations from other sources are not increased and are stable. Because of the multiple sources of origin and the fact that the bone isoform is usually not increased in
osteoporosis and other metabolic bone diseases, total alkaline phosphatase has not enjoyed widespread use as a bone remodeling marker.

Bone, liver, and intestinal isoforms of alkaline phosphatase are posttranslational modifications of the same gene product and can be identified by their unique carbohydrate content (Martin M et al., 1997). Measurement of "fractionated" alkaline phosphatase recognizes that heating destroys the skeletal fraction, which can be determined by subtraction of the stable fraction from the total. This procedure is not sufficiently reproducible to be used clinically. Assays for bone alkaline phosphatase [BAP; also known as bone-specific alkaline phosphatase, or skeletal alkaline phosphatase (SAP)] have been developed using electrophoresis, isoelectric focusing, lectin precipitation, and immunoassay techniques. Immunoassay is the method of choice because of high specificity and satisfactory precision. Commercially available immunoassays have been developed that measure either enzyme activity or mass (Woitge H et al., 1996; Van Straalen JP et al., 1991; Alpers DH et al., 1984). Because BAP is cleared by the liver, the skeletal fraction may be increased in patients with liver diseases. There may also be some cross-reaction of BAP antibodies with liver alkaline phosphatase.

b) Osteocalcin

Osteocalcin, the major noncollagen protein of bone matrix, is a small 49-amino acid protein that is rich in glutamic acid (GLA) (Farley JR et al., 1981). Osteocalcin is also known as bone GLA protein and BGP. In addition to bone, it is also found in dentin. The function of osteocalcin is not clear; it may serve as a site for hydroxyapatite crystals. In the process of matrix synthesis, some osteocalcin is released and circulates in blood with a short half-life determined mainly by renal clearance. Although no intact osteocalcin is released during bone resorption, fragments are released in vitro and also during resorption and formation (Gorman L & Statland BE, 1997; Gallop PM et al., 1980; Hauschka PV et al., 1989). Osteocalcin can be measured by immunoassay in plasma or serum. Osteocalcin is labile in blood. It is reduced in lipemic serum because of binding of osteocalcin to lipids, and osteocalcin may be degraded in vitro by proteolytic enzymes liberated from erythrocytes. Assays for osteocalcin are not
standardized (Gundberg CM et al., 1999), and different antibodies clearly recognize different fragments (Price PA et al., 1987; Ducy P et al., 1996). Antibodies that recognize both the intact molecule and the large N-terminal mid molecule fragment appear to provide the best clinical information (Brown JP et al., 1984)

Although vitamin K status does not affect the total-osteocalcin concentration, it does affect the amount of carboxylation. Undercarboxylated osteocalcin may be a better predictor of certain outcomes such as fracture (Bouillon R et al., 1992; Delmas PD et al., 1983).

c) Procollagen extension peptides
Osteoblasts secrete large procollagen molecules that undergo extracellular cleavage at the amino and carboxy termini. Byproducts of type 1 collagen synthesis are the amino- and carboxy-terminal procollagen 1 extension peptides (PINP and PICP) (Crofton PM, 1992; Gundberg CM et al., 1985; Taylor AK et al., 1988; Monaghan DA et al., 1993; Parviainen M et al., 1994). PINP is an elongated protein of 35 kDa. PICP is a globular protein of 1000 kDa and contains disulfide bonds. Both extension peptides are cleared by the liver. Both may be incorporated into bone matrix. Both can be measured by immunoassay. The concentrations of both increase with increased turnover of nonskeletal collagen (e.g., skin and muscle).

2. BONE RESORBING MARKERS:
Bone resorption markers include an enzyme, tartrate-resistant acid phosphatase (TRAP), and products of bone breakdown, which include calcium and bone matrix degradation products such as hydroxyproline, pyridinium cross-links, and telopeptides. Urinary calcium is affected by diet and renal function and is not sufficiently sensitive or specific for assessment of bone remodeling.

a) TRAP
Acid phosphatase is a lysosomal enzyme found in bone, prostate, platelets, erythrocytes, and spleen. Of the five isoenzymes of acid phosphatase, the bone isoform is tartrate resistant (TRAP) but unstable. TRAP can be measured in
serum or plasma by electrophoresis (after treatment with tartrate) or by immunoassay. Serum acid phosphatase concentrations are typically higher than those in plasma because of the release of acid phosphatase from erythrocytes during clotting.

b) Collagen breakdown products
Type 1 collagen, rich in the amino acid hydroxyproline, has a triple helix structure, with strands connected by cross-links between lysine or hydroxylysine residues that join the non helical amino- and carboxy-terminal ends of one collagen molecule to the helical portion of an adjacent molecule. (Lian JB et al., 1999). The cross-links are pyridinolines and deoxypyridinolines. During bone resorption, hydroxyproline and the pyridinium cross-links may be released either free or with fragments of the collagen molecule attached. They are not reutilized. Although some type 1 collagen is present in non skeletal tissues, bone has a much higher proportion and a much higher turnover.

c) Hydroxyproline
Collagen is rich in the amino acid proline, which undergoes posttranslational hydroxylation to hydroxyproline. Most of the free hydroxyproline liberated from bone is catabolized in the liver; ~10% is released in small polypeptide chains that are excreted in the urine. Hydroxyproline is also liberated by the breakdown of complement and nonskeletal collagen, including dietary collagen, and by the breakdown of procollagen extension peptides, which are products of bone formation. Approximately 50% of urinary hydroxyproline is derived from bone collagen breakdown (Rizzoli R et al., 1999). Hydroxyproline is usually measured in urine by colorimetry or HPLC after hydrolysis to convert peptide and polypeptide forms to the free form.

d) Pyridinium cross-links (pyridinoline and deoxypyridinoline)
Posttranslational modification of lysine and hydroxylysine produces the nonreducible pyridinium cross-links, pyridinoline (Pyr) and deoxypyridinoline (Dpd), that stabilize mature collagen. Both Pyr and Dpd are released from bone in a ratio of approximately 3:1. Dpd is relatively specific for bone; Pyr is also found in articular cartilage and in soft tissues (ligaments and tendons).
Approximately 60% of the cross-links released during resorption are bound to protein, with the remaining 40% being free (not protein bound). Pyridinium cross-links are not metabolized or absorbed from the diet (3). Pyr and Dpd can be measured in urine by HPLC or immunoassay (Stinson RA & Hamilton BA, 1994; Harris H, 1980; Crofton PM, 1982; Koyama I et al, 1987; Langlois MR et al., 1994; Green S et al., 1971) either before or after hydrolysis.

e) Cross-linked telopeptides
In the process of bone resorption, amino- and carboxy-terminal fragments of collagen are released with cross-links attached. These fragments with attached cross-links are called telopeptides. N-telopeptides (NTx) and C-telopeptides (CTx) are excreted in the urine. NTx is measured by immunoassay using an antibody to the α-2 chain of the NTx fragment (which contains the pyridinium cross-links, but the assay does not recognize the cross-link itself) (Van Hoof VO et al., 1990). CTx is measured by immunoassay (Magnusson P et al., 1999). Urine has been the most convenient sample for assay, but efforts have been directed at developing serum assays (Hill CS & Wolfert RL, 1989; Rosalki SB & Foo AY, 1984; Rosalki SB & Foo AY, 1987; Crofton PM, 1992).

f) Cathepsin K
Cathepsin K is a member of the cysteine protease family that, unlike other cathepsins, has the unique ability to cleave both helical and telopeptide regions of collagen I. Its clinical relevance was appreciated with the discovery that pycnodysostosis, an autosomal recessive disease characterised by osteopetrosis, was the result of mutations in the cathepsin K gene. This clinical phenotype has been confirmed in cathepsin K null mice showing dysfunctional matrix digestion. Immunocytochemical studies have shown that cathepsin K is located intracellularly in vesicles, granules and vacuoles throughout the cytoplasm of osteoclasts and that it is secreted into bone resorption lacunae for extracellular collagen degradation. Recently, a new enzyme-linked immunoassay for measurements of cathepsin K in serum has been developed. Due to the fact that cathepsin K is expressed and secreted by osteoclasts during active bone
resorption, cathepsin K, and specifically its circulating form, may be a useful and specific biochemical marker of osteoclastic activity (Seibel MJ, 2005).

**ROLE OF NITRIC OXIDE (NO) IN BONE REMODELING**

NO, an intracellular messenger stimulated by PTH and 1, 25-dihydroxyvitamin D3, has complex effects on bone cells. Earliest studies have linked NO to bone remodeling whereby inhibition of ON synthase (NOS), the enzyme that synthesizes NO from L-arginine, potentiates ovariectomised bone resorption in rats. NO potently suppresses RANKL expression in both primary murine stromal cells and a stromal cell line, ST-2, while up-regulates OPG expression. Low concentrations of NO donors (SNP, NOR-4) have significant effects on both mRNA and protein levels of RANKL and OPG. Besides, NO, other osteoactive factors are known to effect similar co-ordinate changes in RANKL and OPG expression like PTH and IGF-1 stimulates RANKL mRNA expression, while also decreasing OPG expression (Lee and Lorenzo, 2002; Rubin et al., 2002). TGF-α1, similar to NO, decreases RANKL while increasing OPG (Takai et al., 1998). Because of the universality of NO signaling, it may be that some of these factors regulate RANKL/OPG ratios through modulating NOS activity in bone cells. NO can influence their transcription through structural modifications of transcription factors, for instance changing the binding affinities of c-Fos, c-Jun, or NF-kB by S-nitrosylation of cysteines near their DNA-binding domains (Marshall et al., 2000; Nikitovic et al., 1998; Tidball et al., 1999).

NO is constitutively produced in OBs whereby it can be an effective mediator to regulate OB proliferation and differentiation (O'Shaughnessy et al., 2000). However, over expression of NO leads to OB injuries (Damoulis and Hauschka, 1997; Mancini et al., 2000; Mogi et al., 1999). NO can damage OB DNA and induce cell apoptosis (Chen et al., 2004). Besides, SNP, an NO donor, can decrease mitochondrial membrane potential of OBs consequently decreasing cellular ATP levels. SNP enhances release of intracellular ROS and cytochrome c from mitochondria to cytoplasm. Reduction of ATP synthesis (Blom et al., 2003) and enhancement of ROS (Mancini et al., 2000) induce cell apoptosis as is evidenced by suppression of Bcl-2/Bax proteins following SNP treatment of Osteoblasts. Thus, the NO-induced...
death of osteoblastic cells has been shown to occur via an apoptotic pathway (Chen et al., 2004).

**SIGNALING PATHWAYS IN OSTEOCLASTOGENESIS**

Recognition and binding of the RANK by RANKL is the key osteoclastogenic event, inducing a number of parallel signaling cascades. Three important pathways in osteoclastogenesis are described below, which are (a) NF-kB activation pathway, (b) c-Jun signaling pathway and (c) The calcineurin / NFAT signaling pathway. The details are as under:

**a) NF-kB ACTIVATION PATHWAY**

RANK activation by RANKL is followed by its interaction with TNF receptor-associated factors (TRAF) family members TRAF2, TRAF3, TRAF5, and TRAF6 (Wong et al., 1998; Darnay et al., 1998; Darnay et al., 1999; Darnay et al., 1999) which in turn recruits NF-kB inducing kinase leading to the activation of NF-kB (Darnay et al., 1999). TRAF6 and NF-kB play an indispensable role in OC differentiation as demonstrated by the osteopetrotic phenotype of TRAF6 and NF-kB knockouts (Lomaga et al., 1999; lotsova et al., 1997).

NF-kB is a dimer consisting of the NF-kb / Rel family proteins which include p50, p52, p65, c-Rel, and Rel-B. In the unstimulated cells, NF-kB dimer in cytosol is present as an inactive form complexed with an inhibitory protein IkB, two major forms of which are IkB-α (Haskill et al., 1991) and IkB-α (Thompson et al., 1995). When stimulated by stimulatory cytokine, TNF-α, the NF-kB dimer dissociates from IkB and translocates to the nucleus, the process being called ‘activation of NF-kB’. The activated NF-kB dimer binds to the regulatory NF-kB elements in the target genes and regulates their transcription (Baeuerle & Hankel, 1994).
Introduction

Osteoblasts

OPG

TRAF2

RANK

TRAF6

1\alpha,25(OH)_{2}D_{3}

PTH

IL-11

RANKL

Osteoclast progenitors

Mature osteoclasts

Differentiation

M-CSF

Quiescent Osteoclasts

Activated osteoclasts

NF-\kappa B

JNK

Figure (d): A schematic representation of osteoclast differentiation

Osteoclast differentiation supported by RANKL binding with RANK. The RANK signaling is transduced via TNF receptor-associated factor 2 (TRAF2) and TNF receptor-associated factor 6 (TRAF6) leading to the activation of NF-Kb, which in turn stimulates the activation and differentiation of osteoclasts. (Adapted from Udagawa, 2002)

b) c-Jun SIGNALING PATHWAY

RANKL is unique among TNF ligand superfamily members in its capacity to induce OC differentiation. The mechanism probably involves its interaction with TRAF6 (Lomaga et al., 1999). Association of RANKL and TRAF6 activates key events involving activator protein-1 (AP-1) mediated transcription of OC specific genes. The importance of AP-1 transcription factors, especially dimers of the Fos and Jun families of proteins, in the osteoclastogenic process was first documented by Erwin Wager wherein c-Fos knockout mice were shown to be osteopetrotic due to complete absence of OCs (Grigoriadis et al., 1994). RANK
occupancy mobilizes intracellular calcium, a requisite for calcineurin-mediated nuclear factor of activated T cells (NFAT) activation. Moreover, RANKL not only induces the transcription factor’s expression but facilitates its nuclear translocation, where NFAT binds to its DNA response element via a ternary complex with AP-1 proteins, including Fos/Jun, to transactivate target genes (Macian et al., 2001). Thus, RANKL-induced osteoclastogenesis involves partnering of Fos/Jun with NFAT2. NFAT1 functions as the upstream regulator for NFAT2 during osteoclastogenesis and c-Jun is necessary for NFAT1 to induce NFAT2 expression as demonstrated by recent studies that RANK induced NFAT2 expression in bone marrow macrophages (BMM) or RAW264.7 cells promoting their differentiation into TRAP+ osteoclast-like cells even in absence of sRANKL (Ishida et al., 2002; Takayanaki et al., 2002). Besides, over expression of NFAT1, which is expressed in OC progenitors and mature OCs, induces expression of NFAT2 and OC differentiation of RAW264 cells (Ikeda et al., 2004). The partnership between c-Jun/c-Fos and the NFAT family has been shown to be crucial for OC differentiation by enhancement of the osteoclastogenic activity of NFAT1 and NFAT2 by over expression of c-Jun and c-Fos while inhibition by over expression of either dominant negative c-Jun or c-Fos (Ikeda et al., 2004).

(e) **CALCINEURIN / NFAT SIGNALING PATHWAY**

Calcineurin is best known for its role during T cell activation, where it acts to regulate the activity of the nuclear factor of activated T cells (NFAT) family of transcription factors and thereby couples stimulation of the T cell antigen-receptor to changes in the expression of cytokines and other important immunoregulatory genes (Rao et al., 1997; Crabtree et al., 2002). In T cells, calcineurin is activated in response to the T cell receptor-induced increase in the intracellular calcium concentration. Once activated, calcineurin directly dephosphorylates NFAT proteins that are present in a hyperphosphorylated latent form in the cytoplasm and induces their rapid translocation into the nucleus, where in concert with nuclear partner proteins such as activator protein-1 (AP-1) transcription factor complex, they are able to bind cooperatively to their target
promoter elements and activate the transcription of specific NFAT target genes (Rao et al., 1997; Crabtree et al., 2002).

RANK stimulation can activate calcium dependent signaling events via the effects of the Src tyrosine kinase on phospholipase C (Kim et al., 2002). The Src tyrosine kinase is coupled to RANK via its interaction with the cytoplasmic adaptor protein, TRAF-6 (Lomaga et al., 1999). RANK-induced activation of Src is likely to affect the activity of the calcineurin / NFATc1 signaling pathway in two distinct ways. First, the TRAF-6 / Src-dependent activation of phospholipase C and the subsequent production of the calcium mobilizing agent inositol triphosphate lead to an increase in the intracellular calcium concentration which in turn presumably activates calcineurin, thereby inducing dephosphorylation of NFATc1 and its rapid transport into the nucleus. Second, the TRAF-6 / Src-dependent activation of protein kinase B likely results in the phosphorylation and subsequent inhibition of the principal NFATc1 inhibitory kinase, glycogen synthase kinase-3, thereby preventing this kinase from directly phosphorylating active NFATc1 (Beals et al., 1997; Neal and Clipstone, 2001). Enforced expression of an activated NFATc1 allele in RAW264.7 cells is sufficient to minimize the effects of the RANKL stimulation and induce these cells to acquire a morphologically distinct, TRAP-positive, multinucleated osteoclast-like bone resorbing phenotype (Hirotani et al., 2004). cαNFATc1 is also able to induce the expression of a panel of genes including TRAP, cathepsin K, the calcitonin receptor, and the integrin subunits, which together are considered to be molecular markers of fully differentiated Osteoclasts (Teitelbaum, 2002; Suda et al., 1995; Lee et al., 1995; Quinn et al., 1999; Tong et al., 1994)
Figure (e): Signaling cascades during osteoclastogenesis.

Receptor activator of nuclear factor-κB ligand (RANKL)-RANK binding results in the recruitment of tumor necrosis factor receptor-associated factor 6 (TRAF 6), which activates nuclear factor-κB (NF-κB) and mitogen-activated protein kinases. RANKL also stimulates the induction of c-Jun through NF-κB. NF-κB and c-Jun are important for the robust induction of nuclear factor of activated T cells cytoplasmic 1 (NFATc1). RANK signaling further activates calcium dependant signaling via Src tyrosine kinase, the latter of which is critical for the activation and auto amplification of NFATc1. (Adapted from Okamoto & Takayanagi, 2011).

MODULATION OF BONE CELL ACTIVITIES BY TNF-α

TNF-α is a member of the TNF ligand superfamily, and is secreted by many cell types including monocytes/macrophages and OBs. At cellular level, TNF-α modulates a broad spectrum of responses, including inflammation, immunoregulation, proliferation, differentiation, and apoptosis (Ledgerwood et al., 1999). TNF-α induces these responses via two cell-surface receptors termed TNFR1 and TNFR2.
(also called TNFRp55 and TNFRp75, respectively) (Lewis et al., 1991; Tartaglia & Goeddel, 1992). Both receptors transduce intracellular signals that stimulate the proteolytic breakdown of inhibitor of kappa B (IκB), a cytoplasmic inhibitor of NF-κB (Baldwin., 1996; Verma et al., 1995). TNF-α is known to promote bone resorption in vitro and in vivo (Bertolini et al., 1986; Kitazawa et al., 1994; Thomson et al., 1987; Lerner and Ohlin, 1993) and induce secretion of RANKL in osteoblastic cells (Hofbauer et al., 1999). TNF-α also plays an important role in E deficiency induced bone loss in postmenopausal osteoporosis (Kimble et al., 1996; Kimble et al., 1997; Cenci et al., 2000). TNF-α alone or in combination with IL-1 contributes to the increased numbers of OCs seen at sites of bone resorption. Its been recently shown that colony stimulating factors such as IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF) inhibit RANKL-induced OC differentiation by direct action on OC precursors (Khapli et al., 2003; Miyamoto et al., 2001). IL-3 inhibits RANKL-induced OC differentiation, by down-regulation of c-Fos expression and prevention of NF-κB signaling, and diverts the cells to macrophage lineage; whereas, GM-CSF inhibits RANKL-induced OC differentiation by inhibiting c-Fos expression, and diverts the cells to dendritic cell lineage (Yogesha et al., 2005). Using stromal and lymphocyte-free cultures of OC precursors, and whole bone marrow cells, Yogesha and group, show that IL-3 and GM-CSF act directly and irreversibly on OC precursors by significantly inhibiting c-Fms post transcriptionally. Development and expansion of OC precursors is c-Fms dependent. Both colony stimulating factors completely inhibit TNF-α-induced OC formation via action on TNFRs. TNFR1 and TNFR2 differentially impact osteoclastogenesis. It has been reported that TNF-α stimulates OC formation in TNFR2/-/- but fails to stimulate osteoclastogenesis in TNFR1/-/- mice (Abu-Amer et al., 2000; Roggia et al., 2001). IL-3 and GM-CSF have been shown to inhibit OC differentiation by down regulation of mRNA and surface expression of both TNFR1 and TNFR2 in OC precursors as well as whole bone marrow cells suggesting that IL-3 and GM-CSF down-regulate expression of TNFR1 and TNFR2 at transcriptional and translational levels in the presence or absence of neighboring cells (Yogesha et al., 2005).

TNF-α exerts pleiotropic effects on OBs or osteoblast-like cells such as inhibition of DNA and collagen synthesis (Nanes et al., 1989) and osteocalcin gene expression
(Nanes et al., 1991; Kuno et al., 1994) and stimulation of synthesis of proteolytic enzymes such as plasminogen activators and matrix metalloproteinases (Panagakos & Kumar., 1994) and cytokines such as IL-8(Chaudhary et al., 1992). TNF-α increases mRNA levels of IL-6 and ICAM-1 genes in the rat osteoblast-like osteosarcoma cells (ROS 17/2.8 cells) while an antioxidant, N-acetyl cysteine (NAC) inhibits the p50-p65 heterodimer activation of NF-kB indicating that generation of reactive oxygen intermediates (ROI) mediates the TNF-α-dependent activation of NF-kB in ROS 17/2.8 cells. Simultaneously, NAC attenuated the TNF-α-dependent increase in the mRNA levels for both IL-6 and ICAM-1(Kurocouchi et al., 1998). IL-6 stimulates both proliferation and differentiation of the OC precursor cells (Roodman, 1993) and also promotes the B cell proliferation and differentiation, and activate T cells (Hirano et al., 1986; Houssiau & Van., 1992).

MODULATION OF BONE CELL ACTIVITIES BY ESTROGEN

A network of estrogen (E) regulated cytokines is responsible for the changes in bone turnover and the loss of bone induced by E-deficiency. Considerable evidence has accumulated that suggests that Estrogen prevents bone loss by blocking the production of proinflammatory cytokines by bone marrow and bone cells (Pacifici, 1996; Manolagas & Jilka, 1995). The main consequence of increased cytokine production in the bone microenvironment is an increased osteoclast (OC) formation and elongation of their life span (Roodman, 1996). In addition, enhanced cytokine production results in increased activity of mature OCs and an increased osteoblastic activity which compensates only in part the increased rate of bone loss.
Figure (f): Overview of the multiple interactions by which cytokines and estrogen regulate bone resorption.

The arrows indicate stimulatory (↑) or inhibitory (↓) effects of a cytokine on the synthesis of another cytokine or on a particular step in osteoclastogenesis. Estrogen plays a negative effect on the increased cytokine production of IL-1, IL-6 and TNF-α, thus maintaining a healthy bone microenvironment. (Adapted from Pfeilschifter J, 2002)

(a) CYTOKINE PROFILE IN ESTROGEN DEFICIENCY BONE LOSS

Among the cytokines known to be regulated by E are IL-1, IL-6, and TNF (Pacifici, 1996; Manolagas & Jilka, 1995). IL-1 and TNF are the most powerful locally produced stimulators of bone resorption and are well recognized inhibitors of bone formation. IL-1 and TNF activate mature OCs indirectly via a primary effect on osteoblasts (OBs) and inhibit OC apoptosis. Besides, they markedly enhance OC formation by stimulating the proliferation of OC precursors both directly and by enhancing the pro-osteoclastogenic activity of stromal cells. IL-1 and TNF are also powerful inducers of other cytokines that regulate the differentiation of OC precursor cells into mature OCs such as IL-6, M-CSF and GM-CSF. IL-6 is a potent osteoclastogenic factor that exerts its effects via a cell surface receptor that consists of a ligand binding chain (IL-6R)
and a signal transducing chain known as gp130. When bound to soluble IL-6R, IL-6 stimulates the early stages of osteoclastogenesis in human and murine cultures, presumably by forming a complex with gp130 expressed on either stromal cell or OBs (Udagawa et al., 1995). IL-6 is more potent in increasing the formation of OCs from hemopoietic precursors than in activating mature OCs (Lowik et al., 1989; Roodman GD, 1993).

IL-7 is an osteoclastogenic cytokine, the principal sources of which are the bone marrow, stromal cells (Fry & Mackall, 2002) and OBs (Weitzmann et al., 2000). IL-7 promotes osteoclastogenesis by up-regulating key T cell-derived osteoclastogenic cytokines, including receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) (Weitzmann et al., 2000). IL-7 also increases the number of early precursors of the B cell lineage (B220+ cells) in the bone marrow (Miyaura et al., 1997), a population that has been suggested to have the capacity to differentiate into OCs (Sato et al., 2000). Thus, IL-7 may also induce bone loss by increasing the pool of OC precursors. Upregulation of IL-7 which is a consequence of E2 deficiency has been shown to play a central role in ovariectomy-induced bone loss by uncoupling bone formation from bone resorption (Weitzmann et al., 2002) whereby IL-7 treatment of osteoblastic cells led to a decrease in the promoter activity of Cbfa1/Runx2. This ultimately leads to a decrease in the protein concentration of Cbfa1/Runx2 and hence reduced transactivation of Cbfa1/Runx2 responsive promoters. Cbfa/Runx2 is both a marker of and a key participant in the process of OB differentiation. IL-7 stimulates osteoclastogenesis further through down regulation of OPG, the decoy receptor of RANKL, due to reduction of Cbfa1/Runx2 levels. Therefore, IL-7 is a relevant causal agent of bone loss induced by E2 withdrawal, as the stimulatory effect of IL-7 on bone resorption is compounded by the inhibition of bone formation (Weitzmann et al., 2002).
Estrogen deficiency causes a global increment in IL-7 production, especially in thymus, spleen and bone marrow, in turn increasing the T cell pool. T cells produce elevated levels of proinflammatory cytokines including TNF-alpha, IL-1, and IL-6. These cytokines promote increased RANK L expression on osteoblasts and stromal cells, which further leads to osteoclast differentiation in the presence of M-CSF. (Adapted from Zhao R, 2012)

(b) REGULATION OF BONE METABOLISM BY TGF-α & IFN-γ

Estrogen (E) exerts multifactorial control over bone remodeling. Most of the bone-sparing activity exerted by E occurs through modulation of bone cell life-span and decreased cytokine-driven osteoclastogenesis (Kousteni et al., 2001; Riggs et al., 2002). Among the factors that up-regulate OC formation and lead to bone loss in estroprevic humans and rodents is tumor necrosis factor alpha (TNF-α) (Nanes MS., 2003). This E regulated cytokine promotes osteoclastogenesis by augmenting the production of RANKL.
(Pfeilschifter et al., 2002), the non-redundant cytokine responsible for OC development (Hofbauer et al., 2000) and by increasing the responsiveness of maturing OCs to this factor (Cenci et al., 2000; Lam et al., 2000; Zhang et al., 2001). Besides, TNF stimulates the production of other cytokines known to be implicated in the pathogenesis of ovariectomy (ovx) induced bone loss, such as IL-1, IL-6, IL-7, and macrophage colony stimulated factor (M-CSF) (Pfeilschifter et al., 2002). ovx increases TNF levels in the bone marrow (BM) via an expansion of the pool of TNF-producing T cells (Cenci et al., 2000; Roggia et al., 2001) induced by a complex mechanism driven by interferon gamma (IFN-γ) (Cenci et al., 2003). IFN-α augments antigen (Ag) presentation by enhancing MHC D expression on BM macrophages (BMMs), through induction of class D transactivator (CDTA) expression (Boss & Jensen, 2003). Up-regulation of Ag presentation results, in turn, in the increased T cell activation. Thus, up-regulation of IFN-α production induced by ovx leads to the increased T cell proliferation and life-span, a phenomenon that result in an increase in both the total number of T cells and the pool of TNF-producing T cells (Cenci et al, 2003). T cell-produced TNF plays key role in the ovx-induced bone loss, as demonstrated by the failure of ovx to induce bone loss in T cell-deficient nude mice and by the ability to reconstitute with WT T cells, but not TNF-/- T cells, to restore a normal response to ovx (Cenci et al., 2000; Roggia et al., 2001).

TGF-α has been reported to repress the production of IFN-γ by directly targeting T cells and inhibiting their proliferation(Kehrl et al.,1986) and differentiation into effector cells (Gorelik & Flavell, 2002). Also, TGF-α is recognized for its ability to repress the production of and responsiveness to numerous cytokines relevant for bone homeostasis. For example, TGF-α signaling in BMMs decreases the responsiveness of the CDTA gene to IFN-α (Lee et al., 1997; Nandan and Reiner, 1997) via a mechanism involving Smad 3 (Dong et al., 2001), whereas targeting of stromal cells and OBs by TGF-β blunts BM production of IL-7 (Tang et al., 1997). Thus, TGF-α blocks T cell activation and T cell TNF production. Because the levels of TGF-α in bone and serum are increased by E and blunted by ovx (Gray et al., 1989; Finkelman et al., 1992; Bord et al., 2001), TGF-β may be a
pivotal upstream target of E in bone as is suggested by E failure to prevent bone loss in mice with a T cell-specific blockade of TGF-α signaling and complete in vivo prevention of ovx-induced bone loss by overexpression of TGF beta. However still, TGF-β and IFN-α have contradicting effects on bone metabolism. IFN-γ and TGF-β have opposing effects on diverse cellular functions (Lake et al., 1994; Bauvois et al., 1992; Schmitt et al., 1994). IFN-γ signals through the Janus kinase STAT pathway (Stark et al., 1998; Schindler & Darnell., 1995; Ihle JN., 1995) which is generally antagonistic to TGF-β signaling through SMADS-2 and -3 (Massague J., 1998; Heldin et al., 1997) in the regulation of hemopoietic and immune-cell function (Letteria & Roberts., 1998; Ohta et al.,1987). TGF-β has been found to strongly promote TRAP-positive cell formation from mononuclear phagocyte precursors in response to TNF-α, whereas IFN-γ primes these precursor cells for cytocidal macrophage activity (Ding et al., 1988; Russell et al., 1977; Weinberg et al., 1978; Ruco & Meltzer., 1978) while inhibiting TRAP-positive cell formation.

(e) REGULATION OF GLUTATHIONE / THIOREDOXIN AND GLUTATHIONE PEROXIDASE ACTIVITY BY ESTROGEN

Evidence is accumulating that the beneficial effects of E on lipids, endothelial cells, and neurons emanate through improved defense against oxidative stress (Sack et al., 1994; Sudoh et al., 2001). The physiological levels of E are shown to maintain thiol antioxidants in rodent bone marrow as is evidenced by the a substantial decrease in the level of glutathione and thioredoxin, the major tissue thiol antioxidants, and in the enzymes that regenerate their reduced forms in bone marrow of ovx-rats (Jenny et al., 2003). OCs, the bone resorbing cells, is identified as a target for the augmentation of oxidant-defense enzymes by estradiol thereby modulating ROS-sensitive pathways in OCs. Infact, OCs are a prime candidate for regulation of ROS since they express NADPH oxidase (Steinback et al., 1994), an enzyme capable of the cytokine-regulated generation of ROS during bone resorption (Garrett et al., 1990). Besides, the activity of OCs is dependent upon several intracellular signals that are sensitive to ROS, including NF-kB, JNK, P13K, and p38MAP kinase. ROS might also indirectly stimulate OCs by augmenting expression of resorptive cytokines such
as IL-1, IL-6 and TNF-α that have been strongly implicated in E-deficiency bone loss (Jilka et al., 1992; Kitazawa et al., 1994; Roggia et al., 2001). Moreover, ROS and TNF-α not only stimulate resorption but also suppress osteoblastic differentiation (Mody et al., 2001; Gilbert et al., 2002; Panagakos et al., 1996; Samoto et al., 2002). ROS may perturb osteoblastic function by inactivating nitric oxide (NO), deficiency of which is known to impair bone formation in vivo (Armour et al., 2001).

The expression of GPx, the major enzyme responsible for the intracellular degradation of H$_2$O$_2$, modulates the activity of OCs. H$_2$O$_2$ has been shown to directly stimulate OC formation and function (Bax BE et al., 1992; Lean JM. et al., 2003). Bone sparing activity of E has been linked to its ability to induce GPx expression in OCs (Lean et al., 2004). Overexpression of GPx in RAW cells has been shown to abrogate OC differentiation via a suppression of NF-κB signaling pathway which is ROS sensitive. Exposure of cells to H$_2$O$_2$ leads to the induction of expression of cytokines (IL-1, IL-6, TNF-α) implicated in E-deficiency bone loss (Kitazawa et al., 1994; Roggia et al., 2001). H$_2$O$_2$ might also cause bone loss by up-regulation or down-regulation of the expression by bone cells of RANKL or OPG, or through the induction of apoptosis in osteoblastic cells (Kousteni et al., 2002).

**REACTIVE OXYGEN SPECIES (ROS)**

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen and free radicals such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (·OH) and are generated as natural by-products of normal oxygen metabolism. Cells have antioxidant systems, including endogenous enzymes and exogenous substances, that eliminate ROS in order to defend themselves against ROS damage. Increases in ROS production and/or decreases in antioxidant defense can result in significant damage to cell structures, culminating in a situation known as oxidative stress. Since oxidative stress is thought to be a pivotal cause of aging in mammals, a growing body of evidence in human studies suggests that cumulative oxidative damage may be responsible for age-associated bone fragility; however, no causative link has yet been proven definitively (Nojiri H, Saita Y et al., 2011).
PRESENT STUDY

The present study employs a variety of biological effects of allicin are attributed both to its SH-modifying and antioxidant properties (Prasad, K et al., 1995; Ankri, S et al., 1997). L-cysteine of the antioxidant glutathione may also be a target of allicin (Ankri S and Mirelman D, 1999; Rabinkov A et al., 2000). Also, garlic has been reported to scavenge (O₂, H₂O₂ and OH) in a concentration dependent manner (Chaverri JP et al., 2006). Earlier studies involving allicin at higher concentrations have proven to be toxic (Ankri S et al., 1997). Thus, in the present study a lower non-toxic concentration of allicin (500 ng/ml) was used to control the increased TNF-α production due to ROS production, and in turn, a variety of bone markers in culture monocytes as well as to investigate the potential mechanism involved in downregulating TNF-α, which in turn may help in understanding the use of allicin in osteoporosis therapy. The structure of allicin is depicted below in Figure (h). Furthermore, the bioconversion pathway of garlic organosulpher compounds is depicted in Figure (i).

Figure (h): Allicin, IUPAC Name: 2-Propene-1-sulfinothioic acid S-2-propenyl ester; Molecular Formula: C₆H₁₀OS₂ (Adapted from wikipedia.org)
Epigallocatechin gallate (EGCG), also known as epigallocatechin 3-gallate, 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 (e.g. cancer). It is found in green tea, but not black tea; during black tea production, the catechins are converted to theaflavins and thearubigins (Lorenz M and Urban J, 2009). In a high temperature environment, an epimerization change is more likely to occur; however as exposure to boiling water for 30 minutes leads to only a 12.4% reduction in the total amount of EGCG, the amount lost in a brief exposure is insignificant. In fact, even when special conditions were used to create temperatures well above that of boiling water, the amount lost increased only slightly (Wang R et al., 2008). The structure of EGCG is depicted below in Figure (j)
Introduction

Figure (j): Chemical structure of EGCG (Adapted from ikerbill.hubpages.com)

An expanding body of preclinical evidence suggests EGCG, the major catechin found in green tea (Camellia sinensis), has the potential to impact a variety of human diseases. Apparently, EGCG functions as a powerful antioxidant, preventing oxidative damage in healthy cells, but also as an antiangiogenic and antitumor agent and as a modulator of tumor cell response to chemotherapy. Much of the cancer chemopreventive properties of green tea are mediated by EGCG that induces apoptosis and promotes cell growth arrest by altering the expression of cell cycle regulatory proteins, activating killer caspases, and suppressing oncogenic transcription factors and pluripotency maintain factors. In vitro studies have demonstrated that EGCG blocks carcinogenesis by affecting a wide array of signal transduction pathways including JAK/STAT, MAPK, PI3K/AKT, Wnt and Notch. EGCG stimulates telomere fragmentation through inhibiting telomerase activity. Various clinical studies have revealed that treatment by EGCG inhibits tumor incidence and multiplicity in different organ sites such as liver, stomach, skin, lung, mammary gland and colon. Recent work demonstrated that EGCG reduced DNMTs, proteases, and DHFR activities, which would affect transcription of TSGs and protein synthesis. EGCG has great potential in cancer prevention because of its safety, low cost and bioavailability (Singh BN et al., 2011). Reports indicate that
(--)-Epigallocatechin-3-gallate suppresses osteoclast differentiation and ameliorates experimental arthritis in mice (Morinobu A et al., 2008). Thus, in the present study, EGCG has also been employed to probe its effect on human cells from osteoporosis patients.

BACKGROUND
(a) It’s a well established fact that due to augmented generation of reactive oxygen species (ROS) \textit{in vivo} due to a wide spectrum of reasons leads to the activation and up-regulation of bone markers like pro-inflammatory cytokine TNF-alpha and its superfamily member Osteoprotegrin (OPG) as well as calcitonin at both the protein and gene levels.

(b) This in turn results in augmentation in osteoclast differentiation, thereby resulting in bone loss and fractures.

STRATEGY
Thus, in view of the above, if generation of reactive oxygen species (ROS) \textit{in vivo} is arrested by natural antioxidant treatment like allicin and EGCG, then levels of bone markers like TNF-alpha and its superfamily member Osteoprotegerin (OPG) as well as calcitonin will be down-regulated, which in turn will result in suppressed osteoclast differentiation. This in turn would help bones to be healthy and less susceptible to fractures.

Therefore, the present study focuses on Allicin, the main biologically active component of freshly crushed garlic, as well as EGCG from green tea, as a potential agent to counteract the ROS and pathological effects of increased TNF-a production in osteoporosis. Also, allicin and EGCG, as natural antioxidants, has been employed in the present study to regulate bone makers, and in turn, may help in the better understanding in the management of osteoporosis.