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Pre- and Pro-biotics May Improve Mineral Absorption and Retention in the Growing Male Rat

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Abstract

Probiotics are bacteria, which reside in the large intestine and concur beneficial health effects on their host. Their abundance can be selectively-stimulated by prebiotics, such as fructo-oligosaccharide (FOS); prebiotics are oligosaccharides, which are not digested in the small intestine, but pass into the large intestine where they are fermented into short-chain fatty acids. Several studies have suggested that prebiotics may improve mineral absorption. This study aimed to determine the effects of pro- and pre-biotic supplementation on mineral absorption and bone quality in growing male rats.

Sixty three-week old male Sprague-Dawley rats were randomised into five groups and fed either a high-calcium milk powder (HCMP) with or without a probiotic added (groups were subsequently named HCMP – and HCMP + respectively), or HCMP and vitamin K with or without the probiotic (HCMPK – and HCMPK +), or the HCMP with FOS replacing the sucrose in other diets, and the probiotic (the dietary group was named FOS). Animals were maintained on diets for 10 weeks.

Balance studies were carried out during weeks 3 – 4 and 8 – 9 of the study. The earlier balance study suggested that dietary interventions may affect mineral absorption. The latter balance study, however, showed no discernable differences between groups. Several reasons were postulated for this. Active-absorption may have been down-regulated as a result of long-term supplementation, or an increased abundance of probiotics could cause an elevation of nutritional demands. Alternatively, supplementation may not prove beneficial once animals had passed their period of peak absorption. Bone resorption and formation did not appear to have been altered as a result of dietary intervention, when measured after 10-weeks. Bone mineral density and content, calcium, magnesium, zinc and ash contents and bone biomechanical testing also showed no significant differences between dietary groups. Further research is required to determine whether results obtained were due to long-term supplementation and / or due to the joint-supplementation of pre- and pro-biotics.

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Abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
αvβ3	Vitronectin receptor
AKT	Serine-threonine kinase
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenic protein
BMU	Basic multicellular unit
CAII	Carbonic anhydrase II
CaBP	Calbindin
Cbfa-1	Core binding factor α-1
CFU	Colony-forming unit
CTR	Calcitonin receptor
CTx	C-telopeptides of Type I collagen
DEXA	Dual Energy X-ray Absorptiometry
DP	Degree of polymerisation
E	Oestrogen
ERK	Extracellular regulated kinase
FDCR1	Follicular dentritic cell receptor 1 (OPG)
FGF	Fibroblast growth factors
FOS	Fructo-oligosaccharide
GH	Growth hormone
GOS	Galacto-oligosaccharides
HBSS	Hanks Balanced Salt Solution
HCMP	High calcium milk powder
HCMPK	HCMP with added vitamin K
HSH	Hypomagnesemia with secondary hypocalcemia
I	Inulin
ICP-OES	Individually coupled plasma-optical emission spectrometer
IFN- γ	Interferon- γ
IGF	Insulin-like growth factor
IGFBP	IGF-binding protein
IL	Interleukin

JNK	Protein kinase c-Jun N-terminal kinase
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MagT1	Magnesium Transporter protein
Mi	Microphthalmia
MNC	Mononuclear cell
NDO	Non-digestible oligosaccharide
OCIF	Osteoclastogenesis-inhibitory factor (OPG)
ODF	Osteoclast differentiation-inducing factor (RANKL)
OF	Oligofructose
OPG	Osteoprotogerin
Osf-2	Osteoblast-stimulating factor 2 (*Cbfa-1*)
OVX	Ovariectomised
p38 MAPK	Mitogen activated protein kinase
PBS	Phosphate buffer solution
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
RANK	Receptor activator of the NF-κB
RANKL	Receptor activator of the NF-κB ligand
ROS	Reactive oxygen species
RS	Resistant starch
SCFA	Short chain fatty acid
SEM	Standard error of the mean
T_3	Triiodothyroxine
T_4	Tetraiodothyroxine
TGF-β	Transforming Growth Factor β
TNF-α	Tumor necrosis factor-α (cachectin)
TR1 or TNFr1	TNF-receptor-like molecule 1 (OPG)
TRAF6	TNF-receptor associated factor-6
TRANCE	RANKL
TRAP	Tartrate-resistant acid phosphatase

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Introduction

Nutritional recommendations were once based on the amount required by the majority of a population to prevent symptoms of deficiency (within the population one would expect the exact amount required to differ slightly between individuals). More recently, however, the importance of producing recommendations based on the ability of a nutrient to cause or prevent illness in a population has become more established. This has been paralleled by an increase in affluence and hence understanding the effect of an excess intake of a nutrient has also become more significant. As a result, realisation of the potential of nutrition to maximise health and well-being is better appreciated.

One such area in which nutrition may prove useful is in the prevention of osteoporosis. Approximately one in four women, and one in eight women over the age of fifty in New Zealand have low bone density (Sainsbury and Richards, 1997); osteoporosis can lead to a significant reduction in quality of life for sufferers, and high financial costs. Prevention of the disease has so far appeared better than cure; attempts at restoring bone loss have so far proved relatively unsuccessful (Sainsbury and Richards, 1997). Maximising peak bone mass early in life has the potential to reduce the likelihood of osteoporotic fractures later in life. Peak bone mass is, in turn, affected by several factors, such as nutrient availability, exercise and hereditable elements (Sainsbury and Richards, 1997). Improving the efficiency with which the minerals deposited in bone, such as calcium, magnesium and zinc, are absorbed and used in bone has the potential to reduce the morbidity of osteoporosis.

Chapter 1. Literature Review

Section 1. Bone

1.1.1. Function

Bones have five main functions in the body. They provide a frame of support and protect organs and bone marrow. By acting as an attachment site for muscles, bones also permit movement. They are an important storage site of minerals such as calcium and phosphate, whilst the haematopoietic tissue in bone marrow produces blood cells.

1.1.2. Structure

Bones can be classified morphologically into long, short, flat, irregular or round bones. Long bones are long and narrow. Short bones have lengths and widths approximately equal. Flat bones have broad surfaces, and round bones are spherical. The remainder are irregular bones, which have varied shapes.

Each bone is encased in a layer of dense connective tissue that contains blood vessels known as the periosteum. The bone can be divided into three areas, the epiphyses (at the extremities), diaphysis (the shaft), and metaphysis (the growing section, which lies between the epiphysis and diaphysis). A diagram of a long bone showing these anatomical distinctions is shown in Figure 1.

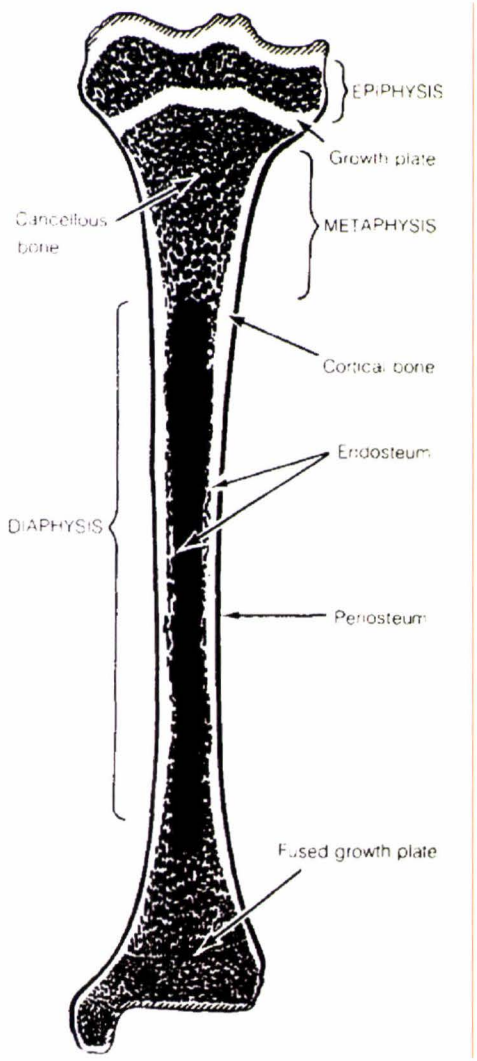

Figure 1: Anatomical features of a long bone, showing epiphysis, metaphysis and diaphysis. Taken from Baron (1999).

Rather than being uniformly solid, bone contains spaces, which provide channels for blood vessels, and reduce the weight of the skeleton. Sections are classified as trabecular (spongy) or cortical (compact), depending on the size and distribution of the spaces they contain, as shown in Figure 2.

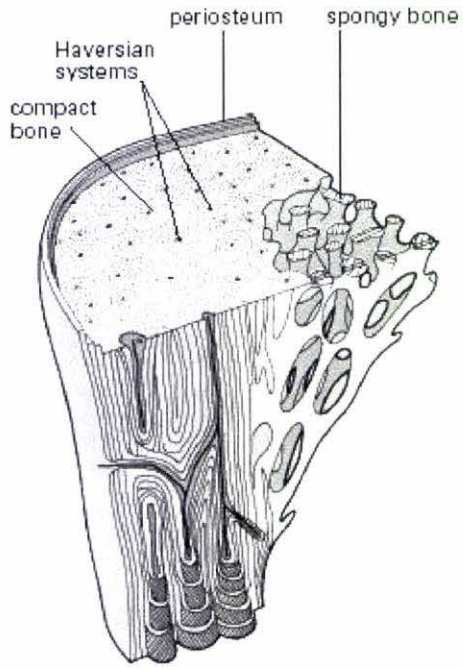

Figure 2: Cortical (compact) and trabecular (spongy) bone. Taken from Oxford University Press (2003).

The relative composition of these two types of skeletal bone differs throughout the body, as shown in Figure 3.

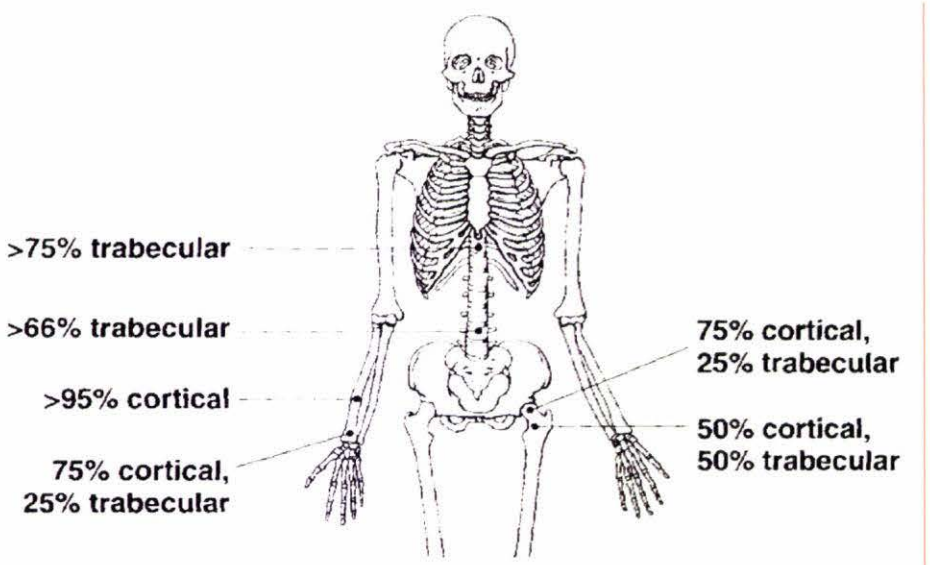

Figure 3: Relative distribution of cortical and trabecular bone in different parts of the skeleton. Taken from Mundy (1999).

1.1.3. Chemical Composition of Bone

There are three main constituents of bone; an organic matrix, inorganic salts, and cells. The bone matrix represents about 30% of the total skeletal mass (Pocock and Richards, 2004). Its major component is collagen; hyaluronic acid and chondroitin sulphate are also present. Bone matrix proteins include osteocalcin, osteonectin, osteopontin, bone sialoprotein, matrix-Gla-protein, fibronectin and alkaline phosphatase. Bone salts are predominately made up of calcium and phosphate in a form known as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). There are three major bone cell types; osteoblasts, osteoclasts and osteocytes.

1.1.3.1. Osteoclasts

Osteoclasts act to resorb bone by demineralisation and degradation (discussed further in 1.1.4.3). A scanning electron micrograph of an osteoclast is shown in Figure 4.

Figure 4: Scanning electron micrograph of an osteoclast. Taken from Shalhoub *et al.* (1999).

Osteoclasts are giant cells formed from mononuclear precursors in the monocyte / macrophage lineage. The presence of certain factors are necessary to signal these precursors to differentiate into the multinucleated osteoclasts rather than other cells in the family, such as erythrocytes, granulocytes, mast cells, megakaryocytes, lymphocytes and macrophages. Some of the factors involved in osteoclast differentiation are shown in Figure 5.

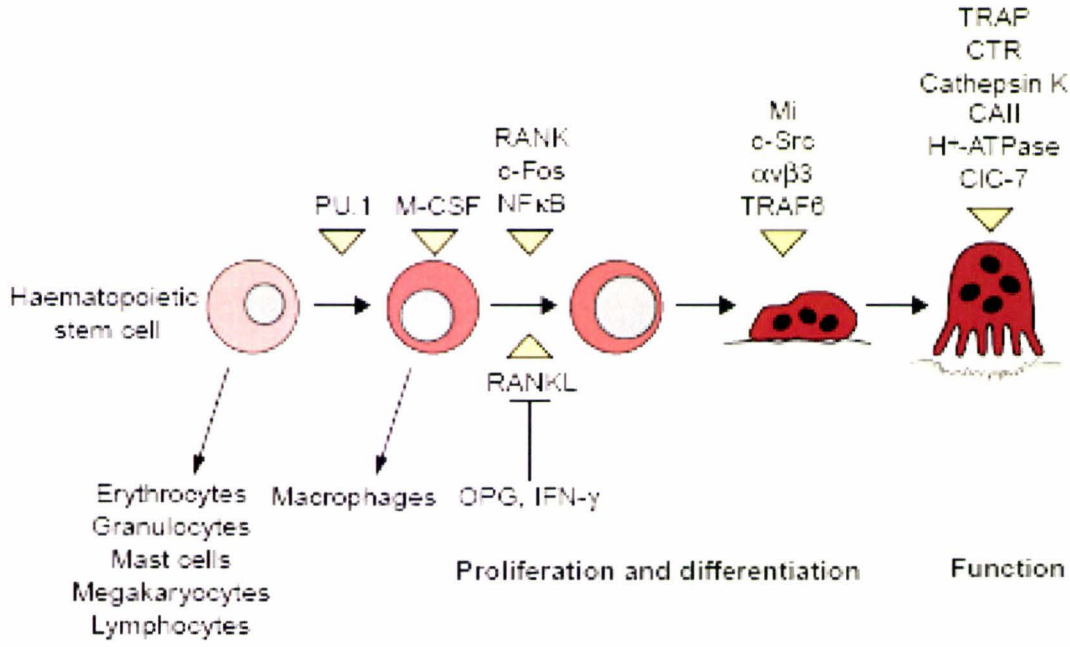

Figure 5: Signals required for osteoclast differentiation and function. αvβ3, Vitronectin receptor; CAII, Carbonic anhydrase II; CTR, Calcitonin receptor; IFN- γ, Interferon- γ; M-CSF, Macrophage colony-stimulating factor; Mi, Microphthalmia; OPG, Osteoprotogerin; RANK, Receptor activator of the NF-κB; RANKL, Receptor activator of the NF-κB ligand; TRAF6, Tumor necrosis factor (TNF) receptor associated factor-6; TRAP, tartrate-resistant acid phosphatase. Taken from Wagner and Karsenty (2001).

PU.1 is a transcription factor that encodes an ETS-domain containing protein required for lymphoid and myeloid differentiation (Wagner and Karsenty, 2001); mice lacking PU.1 are devoid of osteoclasts and macrophages and are osteopetrotic (Tondravi *et al.*, 1997). Osteopetrosis is characterised by increased bone mass and obliteration of bone marrow cavity; bones are dense, brittle and fracture easily (Wagner and Karsenty, 2001; Oxford Reference Online, 2002).

Macrophage-stimulating factor (M-CSF; also known as colony-stimulating factor 1, CSF-1) binds to its receptor c-fms, and, under the influence of the receptor activator of NF-κB ligand (RANKL), signals cells to differentiate into osteoclasts (Zaidi *et al.*, 2005). *op / op* mice are deficient in M-CSF; animals are osteopetrotic, with osteoclasts present in reduced numbers (Chambers, 2000).

RANKL (also known as TRANCE, osteoclast differentiation-inducing factor (ODF), or osteoprotogerin (OPG) ligand) is a member of the tumour-necrosis factor (TNF) family. It is produced as a membrane-bound protein by osteoblasts, and then

cleaved into a soluble form by metalloproteins (Nakashima *et al.*, 2000). RANKL is a protein of 317 amino acids; *OPGL* mRNA is predominately expressed in bone, bone marrow and lymphoid tissues (Steeve *et al.*, 2004). *opgl* mutant mice lack osteoclasts, and show severe osteopetrosis (Kong *et al.*, 1999). RANKL binds to the receptor activator of the NF-κB (RANK), a transmembrane protein of 616 amino acids (Steeve *et al.*, 2004). After binding of RANKL to RANK on osteoclast precursors, the complex interacts with TNF receptor-associated factors (TRAFs) 1 – 6, of which TRAF6 appears to be essential. Mice defective in TRAF6 show osteopetrosis; osteoclasts differentiate, but are unable to resorb bone due to a lack of contact with the bone surface (Lomaga *et al.*, 1999). TRAFs activate several downstream signalling pathways including the NF-κB, AKT (serine-threonine kinase), JNK (protein kinase c-Jun N-terminal kinase), p38 MAPK (Mitogen activated protein kinase) and ERK (extracellular regulated kinase) pathways, which result in osteoclastogenesis, or bone resorption or survival.

Osteoprotogerin (OPG; also known as osteoclastogenesis-inhibitory factor (OCIF), or TNF-receptor-like molecule 1 (TR1 or TNFr1), or follicular dentritic cell receptor 1 (FDCR1)) is a decoy receptor for RANKL. It is also in the TNF-receptor family, and is produced by osteoblasts. OPG is synthesised as a protein of 401 amino acids, and subsequently cleaved to 380 amino acids (Steeve *et al.*, 2004). Its binding to RANKL neutralises the cytokine, inhibiting osteoclastogenesis (Aubin and Bonnelye, 2000; Abu-Amer *et al.*, 2004). OPG deficient mice show increased bone resorption, irrespective of the presence or absence of bone-resorbing factors such as parathyroid hormone (PTH) (Udagawa *et al.*, 1999).

The production of OPG and RANK allows a point of control for osteoclastogenesis. Factors such as Interleukin (IL)-1β, IL-6, IL-11, and TNFα can

promote this process, through increasing expression of RANKL and decreasing expression of OPG, whilst other factors (e.g. IL-13, IL-γ, and TGF-β) can suppress RANKL and / or promote OPG expression, inhibiting osteoclastogenesis (Nakashima *et al.*, 2000).

1.1.3.2. Osteoblasts

Osteoblasts have four main roles in the body; synthesis of components required for the bone matrix, synthesis of factors required for bone formation, regulation of osteoclast activity (through synthesis of factors such as RANKL and CSF-1) and differentiation into osteocytes. A scanning electron micrograph of an osteoblast is shown in Figure 6.

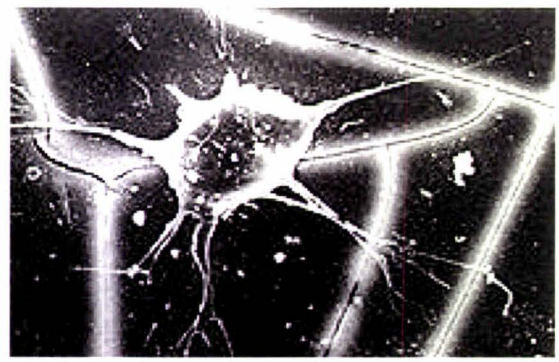

Figure 6: A scanning electron micrograph of an osteoblast. Taken from Loty *et al.* (2001).

Osteoblasts are derived from multipotent mesenchymal stem cell precursors; these precursors also give rise to bone marrow stromal cells, chondrocytes, muscle cells and adipocytes (Manolagas, 2000; Wagner and Karsenty, 2001). The signals required for osteoblast differentiation are shown in Figure 7.

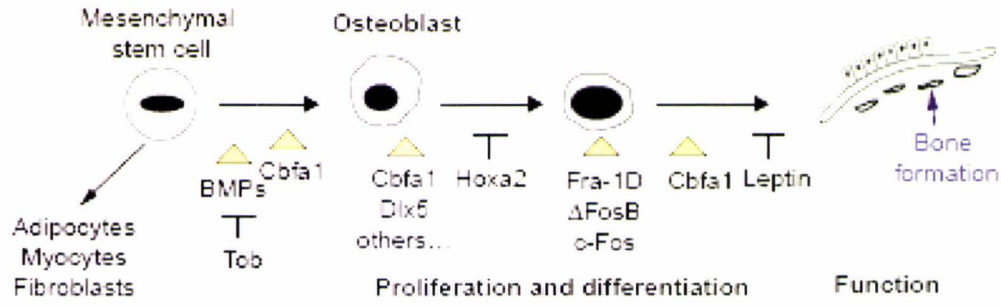

Figure 7: Signals required for osteoblast differentiation and function. Cuboidal osteoblasts are shown on the newly formed bone, together with some osteocytes embedded in the bone matrix. Adapted from Wagner and Karsenty (2001).

Cbfa-1 (Core binding factor α-1; also known as *Runx-2*; or osteoblast-stimulating factor 2, *Osf-2*) is required early in the signalling pathway for osteoblast differentiation. It activates osteoblast-specific genes such as osteopontin, bone sialoprotein, type I collagen and osteocalcin (Ducy *et al.*, 1997; Ducy and Karsenty, 1998; Manolagas, 2000). Deletion of *cbfa-1* results in a complete lack of osteoblasts (Komori *et al.*, 1997; Otto *et al.*, 1997).

Bone morphogenic proteins (BMPs) are members of the Transforming Growth Factor β (TGF-β) superfamily; seven BMPs exist, known as BMPs 1-7, whose roles may overlap (Blair *et al.*, 2002). BMP-2 and BMP-4 are thought to be of particular importance in the differentiation of osteoblasts from their precursors. BMP-4 induces a homeobox-containing gene, distal-less 5 (*Dlx5*), which may act as a transcription factor, regulating the expression of osteocalcin and alkaline phosphatase, as well as regulating mineralisation (Manolagas, 2000). Signalling by BMPs, as with other members of the TGF-β family, involves serine / threonine receptor kinase types I and II. Type I is the signal receptor. To be active it must be associated with the constitutively active type II receptor kinase (Blair *et al.*, 2002). Binding of BMP to types I and II BMP receptors phosphorylates Smad 1, 5 and 8 proteins. These proteins then form a complex with Smad 4 and are translocated to the nucleus, where they interact with other transcription factors such as Cbfa-1 (Chen *et al.*, 2004).

Preventing the downstream signalling caused by BMPs can preclude osteoblast formation (Ghosh-Choudhury *et al.*, 2002); signalling may be regulated at several different levels (Chen *et al.*, 2004).

Insulin-like growth factor (IGF) and fibroblast growth factors (FGF) are also involved in osteoblast differentiation; their role may be limited to acting only on osteoblast progenitor cells that are already committed to this differentiation pathway (Manolagas, 2000). IGF-binding proteins (IGFBPs) can bind to IGFs; some, such as IGFBP-4 are inhibitory, whilst others, such as IGFBP-5 have stimulatory effects (Lian *et al.*, 1999).

1.1.3.3. Osteocytes

Osteoblasts differentiate into osteocytes when the bone matrix surrounds them. The matrix around the osteocytes does not calcify, but forms a lacunocanalicular network between osteocytes, and the surface bone cells, allowing them to remain in contact (Burger and Klein-Nulend, 1999; Tate, 2003), as can be seen in Figure 8. This network is the largest pool of fluid in the bone (Tate, 2003).

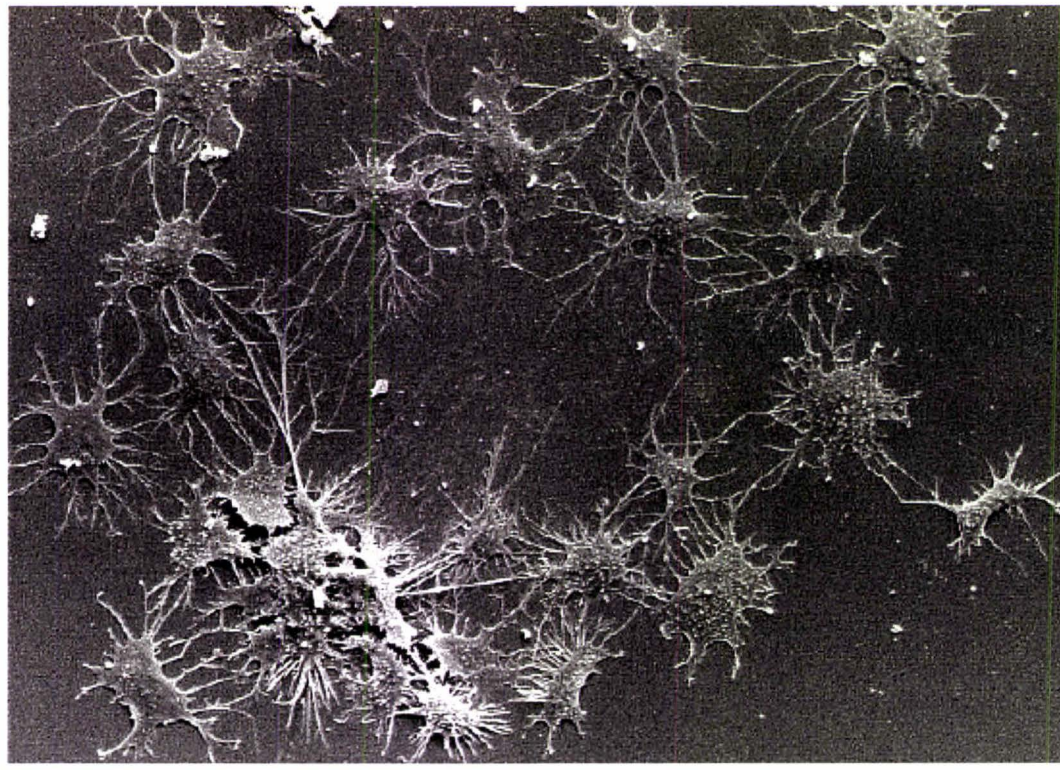

Figure 8: Scanning electron micrograph of osteocytes, isolated from embryonic chicken calvariae, following three days of culture as a monolayer. The lacunocanalicular network can be seen between the osteocytes. Taken from Burger and Klein-Nulend (1999).

1.1.4. Bone Metabolism

1.1.4.1. Ossification

Ossification, or bone modelling, refers to the formation of new bone during embryonic development. There are two types, intramembranous (flat bone) and endochondral (long bone) ossification. In the former, bones are formed directly in connective tissue, whilst the latter also involves cartilage deposition (Oxford Reference Online, 2004).

1.1.4.2. Bone Remodelling

In the adult, bone is continually being broken down and reformed in a process called bone remodelling. Remodelling has two main purposes. It allows bones to become adapted to different levels of stress, in terms of shape and strength, and the replacement of old, degenerating material with new organic matrix. Bone remodelling consists of two processes; bone resorption and bone formation. These procedures occur in the same area of bone, in temporary anatomic structures known as basic multicellular units (BMUs), which has lead to their description as being "coupled". The BMU is approximately 1 – 2 mm long, and 0.2 – 0.4 mm wide. Osteoclasts degrade bone at the front of the unit, and are followed by osteoblasts, which lay down new bone material (Manolagas, 2000). BMUs exist for approximately 6 – 9 months, a period which is split into origination (BMUs commence functioning), progression (advancement of BMUs towards another area of bone requiring replacement) and termination (cessation of BMU functioning) (Manolagas, 2000). In this time, each BMU replaces approximately 0.025 mm^3 of bone (Manolagas, 2000). 3-4 million BMUs are formed each year in the bones of a healthy human, with about 1 million functioning at any one time (Manolagas, 2000).

1.1.4.3. Bone Resorption

The process of bone resorption can be divided into five main stages. These are migration to the resorption site, attachment to the bone surface, establishment of cell polarity, degradation and removal of the bone matrix components, and either osteoclast apoptosis, or their return to the non-resorbing stage (Vaananen *et al.*, 2000; Rousselle and Heymann, 2002). A tightly sealed compartment is formed between the osteoclast and bone surface, isolating the resorption area from the extracellular fluid, which requires $\alpha v\beta 3$ integrin (Blair *et al.*, 2002). Cell polarity is established through the formation of a ruffled border, a specific membrane domain with finger-like extensions that penetrate the bone matrix (Vaananen *et al.*, 2000). Vacuolar H+-ATPases in the ruffled border secrete the H+ ions generated by carbonic anhydrase II (CAII) into the resorption area beneath the osteoclast, facilitating dissolution of the bone matrix (Manolagas, 2000; Rousselle and Heymann, 2002; Martin and Sims, 2005). Matrix metalloproteinases and cathepsin K, secreted by the osteoclast, are also involved in bone matrix degradation (Bossard *et al.*, 1996; Vaananen *et al.*, 2000). The resulting calcium and collagen fragments are then transported by vacuolar transcytosis into the osteoclast (Nesbitt and Horton, 1997; Salo and Lehenkari, 1997). Tartrate-resistant acid phosphatase (TRAP) has been found in these transcytotic vesicles, which generate reactive oxygen species (ROS) able to degrade collagen (Halleen *et al.*, 1999); hence bone matrix degradation may not occur solely extracellularly. This is summarised in Figure 9.

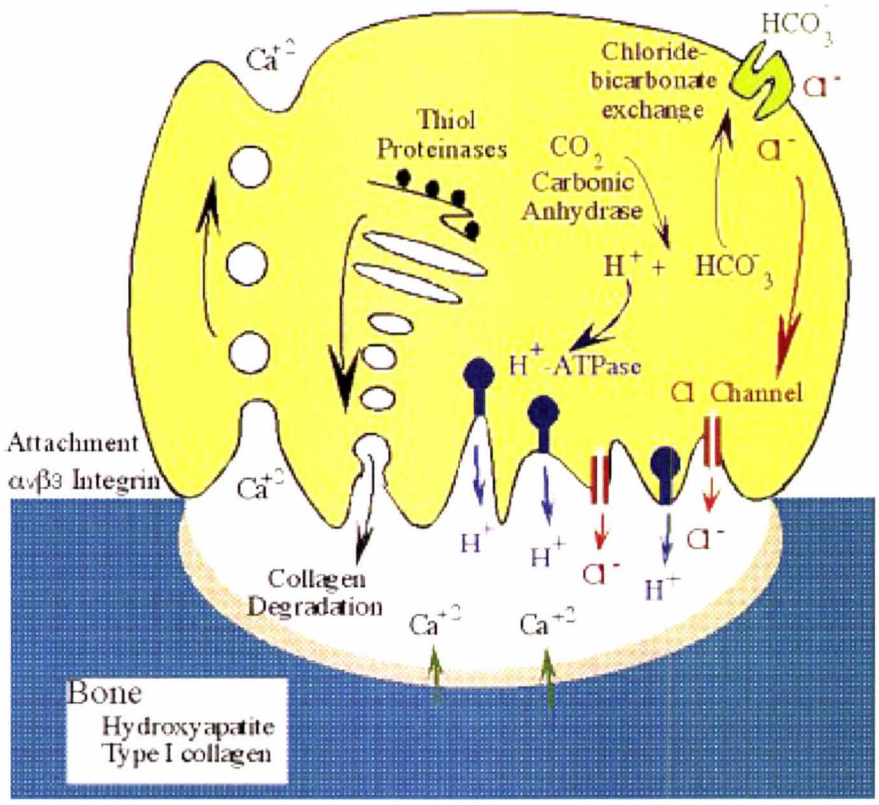

Figure 9: The osteoclast dissolving bone. Taken from Blair (2002).

1.1.4.4. Bone Formation

The activity of osteoblasts in bone formation is less well understood. Bone formation commences with the secretion of the precursor of type I collagen, procollagen, from osteoblasts. These are subsequently cleaved at both the amino- and carboxy-terminal ends, before being subjected to further extracellular processing. The end result are mature three-chained type I collagen molecules that then assemble themselves into a collagen fibril, forming pyridinoline crosslinks with other collagen molecules. Osteoblasts also secrete other proteins that are incorporated into the bone matrix, such as osteocalcin and osteonectin (Manolagas, 2000). They are also responsible for mineralisation (the deposition of hydroxyapatite). This process is not fully understood, but there are two theories as to how it is initiated. The first suggests that the osteoblast produces small vesicles,

which act as nucleation sites for mineralisation, whilst the second suggests that the nucleation site may be the collagen fibril (reviewed by Caverzasio *et al.* (1996)).

1.1.5. Regulation of Bone Metabolism

BMU activity is controlled by the complex interaction of a number of factors. Bone metabolism can be altered in response to lifestyle factors, systemic factors, and local factors, some of which are catabolic, others anabolic. This allows adaptation to environmental conditions and stresses experienced by the individual.

1.1.5.1. Lifestyle factors

Diet and nutrition are the two main lifestyle factors that regulate bone metabolism. Cigarette smoking and high alcohol consumption may be detrimental to bone (World Health Organisation, 2003a). Physical activity can improve bone strength and structure (Marcus, 1999; Brown and Josse, 2002; Kohrt *et al.*, 2004). Overactivity can, however, be detrimental (Brown and Josse, 2002).

Certain nutrients are required for bone metabolism to occur; these include the vitamins A, C, and K, calcium, magnesium and zinc. Vitamin A is involved in the differentiation of osteoblasts; it also decreases collagen synthesis and increases its degradation (Gabbitas and Canalis, 1997; Song *et al.*, 2005). Vitamin C is required for the formation of pyridinoline – deoxypyridinoline crosslinks in collagen molecules (Kipp *et al.*, 1996; Tsuchiya and Bates, 2003; Takamizawa *et al.*, 2004).

Vitamin K is required for the post-translational modification of matrix-Gla-protein and osteocalcin (Yagami *et al.*, 1999; Takeuchi *et al.*, 2000); glutamyl groups on these proteins can be converted to γ-carboxyglutamic acid residues by a vitamin K

dependent γ-carboxylase. These proteins are involved in regulation of bone mineralisation; the carboxylation mediated by vitamin K helps promote calcium binding (Lian *et al.*, 1999). Matrix gla-protein is expressed in many connective tissues, but osteocalcin is more specific to bone. Thus matrix gla-protein and osteocalcin are able to promote cartilage mineralisation and bone mineralisation respectively (Yagami *et al.*, 1999); the proteins may also be involved in the regulation of chondrocyte and osteoclast activity (Lian *et al.*, 1999; Yagami *et al.*, 1999).

Calcium is required for hydroxyapatite crystals in bone mineralisation. Mineralisation provides mechanical rigidity and load-bearing strength to the bone (Lian *et al.*, 1999). Other minerals, such as magnesium and strontium can be incorporated into the crystals should calcium intake be insufficient, but this results in smaller, less perfect crystals, and reduces bone strength (Lian *et al.*, 1999).

Magnesium deficiency has been shown to reduce bone growth, osteoblast number, increase osteoclast number, cause loss of trabecular bone and stimulate productivity or activity of TNF-α, IL-1 and substance P (Creedon *et al.*, 1999; Rude *et al.*, 2003; Rude *et al.*, 2005). Bone quality is reduced, and may even result in osteoporosis (Stendig-Lindberg *et al.*, 2004). Magnesium deficiency is also thought to impair PTH secretion or cause PTH end-organ resistance (Rude *et al.*, 1976) and, therefore hypocalcaemia (Rude *et al.*, 1998). It may also cause reduced serum $1,25(OH)_2D$ (Rude *et al.*, 2005); this may be a result of reduced levels of PTH.

Zinc may improve bone through stimulating bone formation and mineralisation, and inhibition of bone resorption. Zinc may stimulate bone formation and mineralisation through increasing production of alkaline phosphatase, collagen and osteocalcin (Brandaoneto *et al.*, 1995; Cui *et al.*, 1995; Naber *et al.*, 1996). It is

required for DNA and RNA replication, and hence the production of chondrocytes, osteoblasts and fibroblasts (Brandaoneto *et al.*, 1995). Zinc has been shown to inhibit the formation of osteoclast-like cells *in vitro* using mouse and rat bone marrow cells (Yamaguchi and Kishi, 1995; Yamaguchi and Kishi, 1996; Kishi and Yamaguchi, 1997). Zinc supplementation has been shown to improve bone strength in the femoral neck and diaphysis of growing rats (Ovesen *et al.*, 2001).

1.1.5.2. Hormonal factors

1.1.5.2.1. Parathyroid Hormone

Parathyroid hormone (PTH) is a peptide hormone produced by the parathyroid gland in response to a lowering of blood calcium levels. PTH affects both bone resorption and formation (Martin and Sims, 2005). It prevents osteoblast apoptosis and promotes osteoblast differentiation (Dobnig and Turner, 1995; Jilka *et al.*, 1999), but may also act indirectly to increase osteoclast activity (Yu *et al.*, 1996; Greenfield *et al.*, 1999; Swarthout *et al.*, 2002). PTH stimulates the kidney to promote calcium reabsorption and convert inactive 25-hydroxy vitamin D to the active form, 1,25-dihydroxy vitamin D ($1,25(OH)_2D$) (Wood, 2000). Parathyroid hormone-related protein (PTHrP) has effects on osteoclasts identical to those of PTH (Mundy, 1999).

1.1.5.2.2. Vitamin D

$1,25(OH)_2D$ promotes the absorption of calcium from the intestine by increasing the production and activity of several proteins such as calbindin, alkaline phosphatase, low-affinity calcium-dependent ATPase, calmodulin, and brush border actin (Holick, 2000). If dietary availability of calcium is too low to

maintain calcium homeostasis, the vitamin increases bone resorption, by stimulating osteoclastogenesis (Holick, 2000). This ensures that calcium is maintained at a level that allows its passive deposition into hydroxyapatite in bone mineralisation. $1,25(OH)_2D$ can increase transcription of vitamin D-specific genes in osteoblasts, such as osteocalcin, alkaline phosphatase and osteopontin (Holick, 2000).

1.1.5.2.3. Oestrogen

The post-menopausal decrease in circulating oestrogen is well recognised to be responsible for bone loss, and hence potentially cause osteoporosis. The decrease in oestrogen increases the number and activity of osteoclasts, thus increasing bone resorption (Manolagas, 2000; Riggs, 2000). Oestrogen may act to inhibit bone resorption by altering levels of various cytokines, as shown in Figure 10. The increased level of the steroid is also responsible for terminating bone growth at puberty (Gertner, 1999).

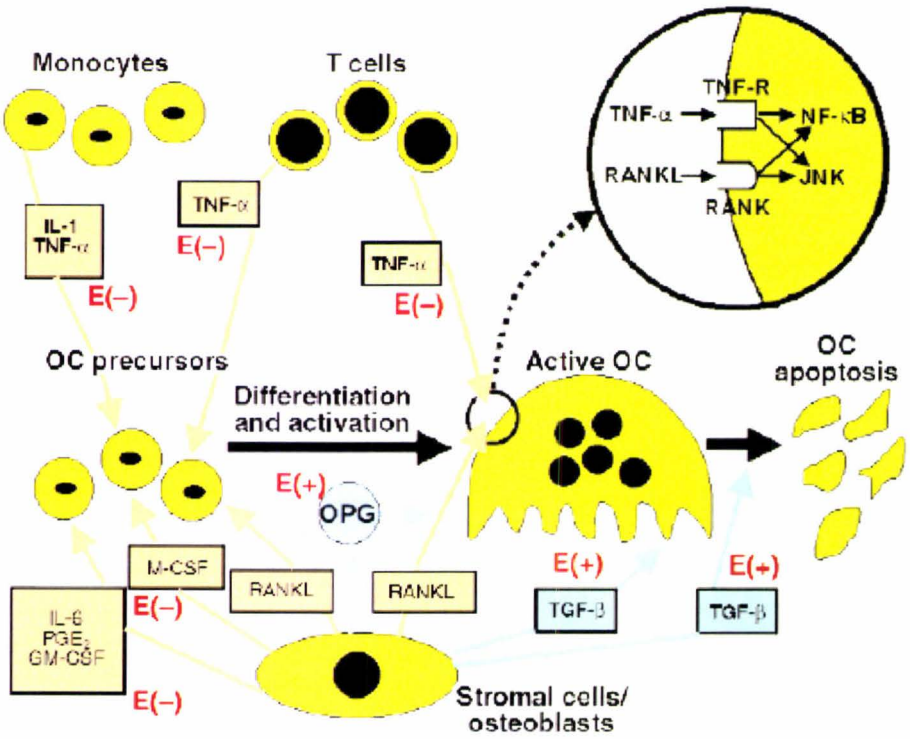

Figure 10: Actions of oestrogen (E) on cytokines in bone. Stimulatory (+) factors are shown in blue and inhibitory (-) effects are shown in orange. Taken from Riggs (2000).

1.1.5.2.4. Growth Hormone

Growth hormone (GH) is a peptide hormone secreted from the pituitary gland, which stimulates release of Insulin-like Growth Factor 1 (IGF-1) from the liver. It stimulates osteoblast proliferation and differentiation both directly, and through IGF-1 (Langdahl *et al.*, 1998; Olsen *et al.*, 2000); this may also indirectly stimulate osteoclast differentiation and activity (Olsen *et al.*, 2000). The overall effect, however, is that of promotion of bone formation.

1.1.5.2.5. Thyroid Hormones

Thyroid hormones are produced in the thyroid gland; predominately in the inactive Tetraiodothyroxine (T_4) form (although some of the active Triiodothyroxine, T_3, is also produced). T_3 promotes long bone growth during development; in adults, excess can cause accelerated bone loss (Bassett and

Williams, 2003). T_3 may stimulate osteoblastic activity both directly and indirectly, through growth factors and cytokines (Bassett and Williams, 2003).

1.1.5.2.6. Insulin

Although insulin is predominately known for its effects on glucose metabolism, it also affects bone metabolism. Insulin promotes bone formation through its actions on osteoblasts, such as alteration of collagen synthesis (Thomas *et al.*, 1998; Ahdjoudj *et al.*, 2001).

1.1.5.2.7. Calcitonin

A high blood calcium level stimulates the release of the peptide hormone calcitonin from the thyroid gland. It acts to decrease the formation and activity of osteoclasts, inhibiting bone resorption (Wood, 2000).

1.1.5.2.8. Glucocorticoids

Glucocorticoids have a multitude of effects on bone. They inhibit calcium absorption from the intestine (thus increasing PTH secretion), and promote calcium secretion from the kidneys, inhibit osteoblast function, and the formation and action of $1,25(OH)_2D$. By decreasing gonadal hormone secretion, and elevated PTH secretion, the number of remodelling sites on bone is increased. Glucocorticoids also stimulate RANKL production, and inhibit RANK production in osteoblasts, promoting osteoclastogenesis and bone resorption (Hofbauer *et al.*, 1999). For example, Cushing's syndrome is characterised by an excess of glucocorticoids, and therefore osteoporosis.

1.1.5.3. Autocrine / Local Factors

As well as the paracrine / systemic factors involved in bone metabolism described above, autocrine / local factors are also produced. Some of these are listed in Table 1.

Table 1: Catabolic and Anabolic Local Factors Involved in Bone Metabolism. Summarised from Watkins *et al.* (2001).

Catabolic Factors (Increase Bone Resorption / Decrease Bone Formation)	
Growth Factors	e.g. EGF, bFGF, FGF-2, PDGF
Cytokines	e.g. TNF, IL-1, IL-4, IL-6, IL-11, M-CSF
Prostaglandins	Particularly PGE2 at high concentrations
Leukotrienes	e.g. LTC4, LTD4, 5-HETE, LTB4 and 12-HETE
Anabolic Factors (Increase Bone Formation / Decrease Bone Resorption)	
Growth factors	e.g. IGF-I, IGF-II, TGF-α, PDGF
Prostaglandins	Particularly PGE2 at low concentrations

TNF-α (tumour necrosis factor-α; cachectin) is a cytokine released by activated macrophages, which stimulates bone resorption and bone cell replication (Lian *et al.*, 1999; Mundy, 1999; Idriss and Naismith, 2000). It also has other roles in the body, including anti-viral, cytostatic and cytolytic actions; it can cause cachexia, suppress erythropoiesis, and may cause signalling events in cells, ultimately resulting in cell apoptosis or necrosis (Lian *et al.*, 1999; Mundy, 1999; Idriss and Naismith, 2000). It appears to be important for resistance to infection and cancers (Idriss and Naismith, 2000). Cenci *et al.* (2000) demonstrated that oestrogen may reduce TNF-α production by T-cells, hence inhibiting bone resorption; oestrogen-deficiency after the menopause results in non-supression of TNF-α levels and ensuing bone loss (Nanes, 2003).

IL-6 is a pro-inflammatory cytokine, which stimulates bone resorption through increasing production of osteoclast precursors (Wang *et al.*, 2003; Xing and Boyce, 2005). IL-6 is also involved in other processes in the body, including immune responses, haematopoiesis, and acute-phase reactions (Simpson *et al.*, 1997). IL-1,

PTH and $1,25(OH)_2D$ promote the expression and release of IL-6 from bone cells (Lian *et al.*, 1999; Mundy, 1999; Riggs, 2000).

IL-10 is an anti-inflammatory cytokine produced by T-cells and macrophages; it induces a wide range of biological activities in the body, such as preventing cytokine production, increasing survival of T- and B-cells and the activity of NK cells (Pestka *et al.*, 2004). Mice deficient in IL-10 show chronic inflammation of the intestine, as they are unable to control immune responses to intestinal flora (Kuhn *et al.*, 1993). Production of IL-10 may be induced by TNF-α in macrophages, lipopolysaccharide (LPS), and IL-6 and IL-12 in T-cells (Daftarian *et al.*, 1996). The cytokine also has anabolic effects on bone (Daftarian *et al.*, 1996; Watkins *et al.*, 2001; Stenvinkel *et al.*, 2005). These effects are due to its inhibition of osteoclast formation, achieved through a direct action on osteoclast precursors (Hong *et al.*, 2000).

SUMMARY

Bones provide support, protection, permit movement, produce blood cells, and act as a storage reservoir for certain nutrients. There are two types of bone, cortical and trabecular; the body is composed of different percentages of these two types of bone allowing greater adaptation in terms of weight, strength, and resource allocation. Alternatively, bones can be classified chemically; bones are composed of an organic matrix, inorganic salts and bone cells (osteoblasts, osteoclasts and osteocytes). Differentiation of these bone cells is controlled by several different factors. In order to develop and function correctly, osteoclasts require PU.1, M-CSF, RANK, and RANKL, amongst other factors. OPG can bind RANKL, a decoy receptor, preventing

osteoclastogenesis. Osteoblasts, however, require *Cbfa-1*, BMPs, IGFs and FGFs in order to function correctly.

There are two processes of bone growth that occur; bone modelling (ossification) and bone remodelling. Ossification allows bones to grow in size, and occurs from the start of life up until the end of adolescence. Bone remodelling, however, occurs throughout life, allowing bones to become adapted to different levels of stress, and the replacement of old material. Remodelling consists of two processes, bone resorption and bone formation; these are regulated by the complex interaction of several hormones and cytokines on activity of BMUs. PTH, Vitamin D, oestrogen, GH, insulin, and calcitonin promote bone formation, whilst thyroid hormones and glucocorticoids promote bone resorption. Several local factors are also produced, including the cytokines IL-6 and TNF-α, which increase bone resorption, and IL-10, which increases bone formation. An imbalance of these factors may have serious consequences, resulting in bone overgrowth, causing problems for nerve and blood supply, or bone weakening, increasing fracture risk