

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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

**A thesis presented in partial fulfilment of the
requirements for the degree of**

**Master of Science
In
Nutritional Science**

**At Massey University, Turitea,
Palmerston North,
New Zealand.**

**Alison Lindsay Fear
2005**

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.

Acknowledgements

I should very much like to thank the following people, without whom this thesis would not have been possible, and whose personalities and wealth of knowledge made it all the more enjoyable.

- My supervisors Assoc. Professor Marlena Kruger and Dr. Wei-Hang Chua for their time, knowledge, patience and ability to explain statistics.
- The Institute of Food, Nutrition and Human Health (Massey University), in particular:
 - Mrs Chris Booth – for her guidance, experience and patience
 - Mrs Anne Broomfield – for her kiwi ingenuity and practical help
 - Dr. Jane Coad – for her continual support and faith over the past few years
 - Miss Linley Fray – for teaching me about interleukins
 - Miss Hilary McKinnon – for her help and sense of humour during the darker moments
 - Miss Raewyn Poulsen – for knowing answers to a wide range of questions
 - Mrs Kim Wylie – for enjoying dissection and being at SAPU
- Fonterra Research Centre and Massey University Nutrition Laboratory for their analytical work.

I should like to thank Fonterra Brands Ltd for funding the study, and finally New Zealand Commonwealth Scholarships and Fellowships Committee, who provided financial support in the form of a Commonwealth Masters Scholarship. I am forever indebted to you for believing in me when others may not have.

Abbreviations

1,25(OH) ₂ D	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
<i>Cbfa-1</i>	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 dendritic 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
<i>Osf-2</i>	Osteoblast-stimulating factor 2 (<i>Cbfa-1</i>)
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- κ B
RANKL	Receptor activator of the NF- κ B 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

Table of Contents

Abstract	i
Acknowledgements	ii
Abbreviations	iii
List of Figures	viii
List of Tables	xii
Introduction	1
Chapter 1. Literature Review	2
Section 1. Bone	2
1.1.1. Function	2
1.1.2. Structure	2
1.1.3. Chemical Composition of Bone	5
1.1.3.1. Osteoclasts	5
1.1.3.2. Osteoblasts	8
1.1.3.3. Osteocytes	10
1.1.4. Bone Metabolism	12
1.1.4.1. Ossification	12
1.1.4.2. Bone Remodelling.....	12
1.1.4.3. Bone Resorption.....	13
1.1.4.4. Bone Formation.....	14
1.1.5. Regulation of Bone Metabolism	15
1.1.5.1. Lifestyle factors.....	15
1.1.5.2. Hormonal factors.....	17
1.1.5.2.1. Parathyroid Hormone	17
1.1.5.2.2. Vitamin D.....	17
1.1.5.2.3. Oestrogen	18
1.1.5.2.4. Growth Hormone	19
1.1.5.2.5. Thyroid Hormones	19
1.1.5.2.6. Insulin.....	20
1.1.5.2.7. Calcitonin	20
1.1.5.2.8. Glucocorticoids	20
1.1.5.3. Autocrine / Local Factors.....	21
SUMMARY	22
Section 2. Mineral Absorption	24
1.2.1. Absorption.....	24
1.2.1.1. Calcium	25
1.2.1.2. Magnesium.....	28
1.2.1.3. Zinc	29
1.2.2. Mineral Balance	31
1.2.3. Bioavailability	33
SUMMARY	35
Section 3. Pre- and Pro-biotics.....	37
1.3.1. Pre- and Pro-biotics.....	37
1.3.2. Roles in Mineral Absorption.....	38
1.3.2.1. Calcium	38
1.3.2.2. Magnesium.....	41
1.3.2.3. Zinc	43
1.3.3. Effects on Bone.....	45
SUMMARY	46
Section 4. Motivation and Objectives	48

1.4.1. Motivation for the Study	48
1.4.2. Objectives.....	48
1.4.3. Hypothesis.....	48
Chapter 2. Materials and Methods	49
2.1. Materials.....	49
2.2. Methods.....	49
2.2.1. Animals	49
2.2.2. Diets	50
2.2.3. Balance Studies	52
2.2.4. Terminal Heart Puncture.....	54
2.2.5. Dual Energy X-ray Absorptiometry Scans	55
2.2.6. Biochemical Markers	55
2.2.6.1. Type I Collagen c-Terminal Telopeptides	55
2.2.6.2. IL-6 and IL-10.....	55
2.2.7. Biomechanics	56
2.2.8. Bone Ash Content	56
2.2.9. Gut Bacteriology	57
2.2.10. Statistical Analysis.....	58
2.3. The Growing Rat as a Model	59
2.4. Dual Energy X-ray Absorptiometry.....	60
2.5. Biochemical Markers of Bone Metabolism	61
2.5.1. Type I Collagen c-Terminal Telopeptides	62
2.5.2. IL-6 and IL-10.....	62
2.6. Biomechanical Testing.....	63
SUMMARY	65
Chapter 3. Results	66
3.1. Animals	66
3.2. Diets	67
3.2.1. Food Intake	67
3.2.2. Mineral Content	69
3.2.3. Probiotic Content	69
3.3. Balance Studies.....	70
3.3.1. Mineral Balance	70
3.3.2. Urinary Mineral Content.....	76
3.3.3. Faecal Mineral Content	79
3.3.4. Faecal Weight	81
3.4. Blood / Serum Analysis	84
3.4.1. Type I Collagen c-Terminal Telopeptides	84
3.4.2. IL-6.....	84
3.4.3. IL-10.....	85
3.5. Bone Analysis	85
3.5.1. Dual Energy X-ray Absorptiometry Scans	85
3.5.2. Bone Biomechanics.....	87
3.5.3. Bone Ash Content	92
3.5.4. Bone Mineral Content.....	95
3.6. Gut Bacteriology	96
Chapter 4. Discussion	98
4.1. Mineral Balance	98
4.1.1. Effect of Probiotic Supplementation.....	98
4.1.2. Effect of Vitamin K Supplementation.....	100

4.1.3. Effect of Prebiotic Supplementation	102
4.2. Faecal Weight	105
4.2.1. Effect of Probiotic Supplementation.....	105
4.2.2. Effect of Prebiotic Supplementation	106
4.3. Type I Collagen c-Terminal Telopeptides	107
4.4. IL-6 and IL-10.....	108
4.5. Bone Mineral Density	109
4.6. Biomechanical properties of bone.....	111
4.7. Bone Ash Content	113
4.8. Bone Mineral Content	113
4.9. Gut Bacteriology	114
4.10. Context	115
SUMMARY	116
References	120

List of Figures

Figure 1: Anatomical features of a long bone, showing epiphysis, metaphysis and diaphysis. Taken from Baron (1999).....	3
Figure 2: Cortical (compact) and trabecular (spongy) bone. Taken from Oxford University Press (2003).....	4
Figure 3: Relative distribution of cortical and trabecular bone in different parts of the skeleton. Taken from Mundy (1999).....	4
Figure 4: Scanning electron micrograph of an osteoclast. Taken from Shalhoub et al. (1999).	5
Figure 5: Signals required for osteoclast differentiation and function. $\alpha v\beta 3$, Vitronectin receptor; CAII, Carbonic anhydrase II; CTR, Calcitonin receptor; IFN- γ , Interferon- γ ; M-CSF, Macrophage colony-stimulating factor; Mi, Microphthalmia; OPG, Osteoprotegerin; RANK, Receptor activator of the NF- κ B; RANKL, Receptor activator of the NF- κ B ligand; TRAF6, Tumor necrosis factor (TNF) receptor associated factor-6; TRAP, tartrate-resistant acid phosphatase. Taken from Wagner and Karsenty (2001).	6
Figure 6: A scanning electron micrograph of an osteoblast. Taken from Loty et al. (2001).	8
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).	9
Figure 8: Scanning electron micrograph of osteocytes, isolated from embryonic chicken calvariae, following three days of culture as a monolayer. The lacunocanicular network can be seen between the osteocytes. Taken from Burger and Klein-Nulend (1999).	11
Figure 9: The osteoclast dissolving bone. Taken from Blair (2002).....	14
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).	19
Figure 11: Graph showing net absorption against intake of a hypothetical mineral. Kinetics of absorption are those that could be expected if absorption took place using solely a transcellular active pathway (shown in red), a paracellular passive pathway (shown in blue) or a combination of the two (shown in yellow). The normal physiological range of magnesium intake (represented by the grey bar) is shown to allow a reference point for the importance of each transport mechanism. Adapted from Konrad et al. (2004) and Fleet (2000).....	24
Figure 12: Routes of calcium absorption. Calcium is represented by red circles. The channel CaT2, and transport protein CaT1 absorb calcium from the intestinal lumen. Intracellular transport occurs using CaBP. Calcium is extruded from the cell by CaATPase. This is known as the transcellular pathway. Alternatively, calcium can be absorbed by the paracellular pathway, passing directly into the blood.....	27
Figure 13: Routes of magnesium absorption. Magnesium is represented by green circles; sodium by purple circles. Intracellular transport is thought to occur through the magnesium channel TRPM6. The method of extrusion remains to be determined,	

although it has been suggested that a magnesium / sodium antiporter may be involved. This constitutes the transcellular pathway. The paracellular pathway absorbs magnesium directly into the blood. Adapted from Konrad et al. (2004).29

Figure 14: Current understanding of the method of zinc absorption from the small intestine. Zinc is represented by blue circles. Zinc is thought to enter enterocytes by the ZIP transporters, and be extruded by the ZN-T transporters. Method of transport between the apical and basolateral membranes remains to be determined. This constitutes the transcellular pathway. The paracellular pathway absorbs zinc directly into the blood..... 31

Figure 15: Routes of entry into and exit from mineral pool in the body. The pool consists of the mineral in solution of blood plasma, extracellular fluid and bone. Entry is from absorption from food and resorption from bone. Calcium, magnesium and zinc are excreted in urine, used in bone formation or excreted in faeces. Miscellaneous losses comprise those in sweat, semen, menstrual fluid and breast milk. Adapted from Bronner and Pansu (1999). 32

Figure 16: Mechanisms by which NDOs may increase calcium absorption. Cell A shows how short chain fatty acids (SCFAs) can alter electrolyte exchanges. Cell B shows that SCFA may also form complexes with calcium that are more soluble than calcium ions and therefore more readily absorbed. Cell C represents the possible increase in CaBP resulting from NDOs; NDOs may also cause epithelial atrophy, increasing surface area over which calcium can be absorbed. Although not depicted, the increased production of SCFA can cause calcium in the lumen to become ionised, thus making it available for absorption. 41

Figure 17: Calculation of recovery coefficient 53

Figure 18: Balance calculations 54

Figure 19: Serial dilution, culture and counting of caecal bacteria. Caecal contents were weighed, and diluted (by a factor of ten) in broth in eppendorf 1. 0.1ml of this solution was then diluted in 0.9ml broth in eppendorf 2. 0.1ml of each dilution was also plated out and incubated in anaerobic conditions. This dilution process was repeated until the approximately 30 – 300 colonies were visible; plate A (and all plates prior to plate A, none of which are shown for ease of viewing) would have too many colonies, and plate C too few. Numbers below the tubes show the concentration at which cells are present, relative to the original sample. By aliquoting only 0.1ml onto each plate, cells are in effect diluted again by a factor of ten. Therefore, since in this example plate B shows 36 CFU, this suggests that the original caecal contents would be 36×10^{-6} CFU, or 3.6×10^{-5} CFU..... 58

Figure 20: Bone calcium deposition rate (expressed on a body weight basis) changes with age (graph A), and bone mineral content changes with age in females (graph B) and males (C). Taken from Bronner and Pansu (1999)..... 60

Figure 21: Typical load-deformation curve. Load is a measure of force applied, deformation a measure of amount of bending the bone displays. The red section indicates the elastic phase, the yellow the plastic phase, and the blue section represents the bone break point. Terms are summarised in the text..... 64

Figure 22: Weekly changes in rat body weights during trial. Animals are classified by dietary groups (twelve animals per group), in order to determine that there was no difference induced as a result of diet, as this may have affected nutrient absorption

and / or bone parameters measured. Rats were aged 5-weeks at the start of the trial (week 1), and 14-weeks at the end of the trial (week 10).	66
Figure 23: Mean daily food intake of diet groups during trial. The red line (maximum) shows the amount of food that was given to animals each day. Animals are classified by dietary groups; there were twelve rats in each dietary group. Rats were aged 5-weeks at the start of the trial (week 1), and 14-weeks at the end of the trial (week 10).	68
Figure 24: Fractional absorption (graphs A, C and E) and absolute absorption (graphs B, D, and F) of calcium (graphs A and B), magnesium (graphs C and D) and zinc (graphs E and F) in first balance study, following three weeks of feeding trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group.....	71
Figure 25: Effect of diet on fractional and absolute absorption of calcium, magnesium and zinc in first and second balance studies. The first balance study was carried out after three weeks, and the second after nine weeks of receiving trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group in the first balance study, and twelve in each dietary group in the second balance study.....	74
Figure 26: Comparison of urinary calcium (Ca) magnesium (Mg) and zinc (Zn) excretion in first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group.....	78
Figure 27: Comparison of calcium (Ca) magnesium (Mg) and zinc (Zn) excretion in faeces, and faecal weight during first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group.....	80
Figure 28: Effect of food intake (graphs A and C) and body weight (graphs B and D) on dry faecal weight. Graphs A and B show data from the first balance study (after three weeks on trial diets); C and D show data from the second balance study (after nine weeks on trial diets). There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group.....	82
Figure 29: Comparison of effect of diet of faecal weight (g/day) in first and second balance studies. Animals are classified by the diets they received. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group. The first balance study was carried out after three weeks, and the second after nine weeks of receiving trial diets.....	83
Figure 30: Effect of body weight on spine and femur area, bone mineral content (BMC) and bone mineral density (BMD). Measurements were made using ex vivo DEXAs after 10 weeks of receiving trial diets, on sixty rats.....	86
Figure 31: Relationship between rat weight, femur weight and length and bone biomechanical parameters (maximum load, maximum stroke, break stroke and break strain). Measurements were made on femurs of sixty rats who had consumed trial diets for ten weeks. Measurements were made after ten weeks of receiving trial diets.	89

Figure 32: Relationship between rat weight, femur weight and length and bone biomechanical parameters (break strain, elasticity, and energy required to break bones). Measurements were made on femurs of sixty rats who had consumed trial diets for ten weeks. 90

Figure 33: Relationship between rat weight and bone (femur) length and parameters measured during ashed bone analysis (dry weight, ashed weight and non-mineral bone matter). Measurements were made on sixty rats who had consumed trial diets for ten weeks. 94

Figure 34: Graph showing the differences caused by diets on the colony forming units (CFUs) per gram of caecum. Caecal counts were made from rats who had been consuming trial diets for ten weeks; there were twelve animals per dietary group. Data are classified by dietary groups of the animals. 97

Figure 35: Diagrammatic representation of the bone-mass changes during the life-time of an individual who achieves their full genetic potential, and one who does not (magnitude of differences between these two is not to scale). If both individuals experience the same drop in bone mass later in life (as a result of menopause etc), the person with lower bone mass will reach a level of high fracture risk sooner. Underneath the graph are written several of the factors known to be of particular importance in determining bone mass. Taken from Heaney (2000). 112

List of Tables

Table 1: Catabolic and Anabolic Local Factors Involved in Bone Metabolism. Summarised from Watkins <i>et al.</i> (2001).	21
Table 2: Tissue expression of the nine zinc transporters found in research carried out so far. Where tissues are followed by (?) more research is needed. Summarised from Liuzzi and Cousins (2004).	30
Table 3: Beneficial effects of predominant bacteria in the large intestine of humans. Bacteria in light blue may also have pathogenic effects. Summarised from Gibson and Roberfroid (1994).	37
Table 4: Summary of previous research investigating the effect of prebiotics on calcium absorption. ns indicates no significance; * P<0.05; ** P<0.005. Abbreviations used: FOS, fructooligosaccharides; GOS, Galactooligosaccharides; I, inulin; OF, oligofructose; OVX, ovariectomised; RS, resistant starch.	39
Table 5: Summary of previous research investigating the effect of prebiotics on magnesium absorption. ns indicates no significance; * P<0.05; ** P<0.005. Abbreviations used: FOS, fructooligosaccharides; I, inulin; OF, oligofructose; OVX, ovariectomised; RS, resistant starch.	42
Table 6: Summary of previous research investigating the effect of prebiotics on zinc absorption. ns indicates no significance; * P<0.05; ** P<0.005. Abbreviations used: FOS, fructooligosaccharides; I, inulin; OF, oligofructose; RS, resistant starch.	44
Table 7: Analysis of carbohydrates present in FOS (Frutafit®)	49
Table 8: Diet composition expressed as grams per kilogram	51
Table 9: Summary of trial proceedings, and rat ages.	52
Table 10: Definitions of parameters measured in 3-point bending test.	65
Table 11: Analysis of total food eaten by animals on different diets; there were twelve rats per dietary group. Values with different superscripts (a) denote significant differences between groups at p<0.05.	68
Table 12: Analysis of calcium, magnesium and zinc levels of study diets; four samples of each diet were taken. Values with different superscripts (a) denote significant differences between groups at p<0.05.	69
Table 13: Probiotic counts of the original sample (given as colony forming units (CFUs) / gram) and the three probiotic diets (given as CFU / day). Four samples of each diet were taken.	70
Table 14: Fractional and absolute absorption of calcium (Ca), magnesium (Mg) and zinc (Zn) in first balance study, following three weeks of feeding trial diet. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group. Values with different superscripts (a, b, c) denote significant differences between groups at p<0.05 on the same row.	72
Table 15: Absolute and fractional absorption of calcium (Ca), magnesium (Mg) and zinc (Zn) in second balance study, following nine weeks of feeding trial diet. There were twelve animals in each dietary group. Values with different superscripts (a) denote significant differences between groups at p<0.05 on the same row.	73

- Table 16: Comparison of calcium (Ca), magnesium (Mg) and zinc (Zn) absorption in first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks of receiving trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group. Significance is displayed by sig. NS indicates no significance difference between the two balance studies, * indicates significance of $p < 0.05$ and ** indicates significance of $p < 0.01$ 75
- Table 17: Calcium (Ca), magnesium (Mg) and zinc (Zn) (mg/day) excreted in urine in first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group. Different superscripts (a,b) denote statistical differences between groups on the same row at $p < 0.05$ 76
- Table 18: Comparison of urinary calcium (Ca) magnesium (Mg) and zinc (Zn) excretion in first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group. NS indicates no significance, * indicates significance of $p < 0.05$, and ** indicates significance of $p < 0.01$ 77
- Table 19: Calcium (Ca), Magnesium (Mg) and Zinc (Zn) content of faeces, and faecal weight, from animals receiving diets for three weeks (first balance study) and nine weeks (second balance study). There were eleven animals in each of the HCMP +, HCMPK - and HCMPK + groups, ten in the HCMP - group and twelve in the FOS group. Values with different superscripts (a, b) denote significant differences between groups on the same line at $p < 0.05$ 79
- Table 20: Comparison of calcium (Ca), magnesium (Mg) and zinc (Zn) faecal excretion, and faecal weight in the first and second balance studies; the first balance study was carried out after three weeks and the second after nine weeks of receiving trial diets. There were eleven animals in each of the HCMP +, HCMPK - and HCMPK + groups, ten in the HCMP - group and twelve in the FOS group. Significance is displayed by sig. NS indicates no significance difference between the two balance studies, * indicates significance of $p < 0.05$ and ** indicates significance of $p < 0.01$. 81
- Table 21: Pearson's correlation coefficients and p-values for the effect of food intake and rat weight on faecal weight (g/3days), in first (after two weeks on trial diets) and second balance studies (after nine weeks on trial diets). There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group. ** indicate significance of $p < 0.05$ 81
- Table 22: Analysis of faecal weight in first and second balance study, after three and nine weeks respectively, on trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and twelve in the FOS group. Values with different superscripts (a, b, c) denote significant differences between groups on the same row, at $p < 0.05$ 83
- Table 23: Comparison of faecal weight in first and second balance studies between animals consuming different diets. The first balance study was carried out after three weeks, and the second after nine weeks of receiving trial diets. There were eleven animals in each of the HCMP -, HCMP +, HCMPK - and HCMPK + groups, and

twelve in the FOS group. NS indicates no significance and ** indicates significance of p<0.01.	84
Table 24: Serum Type I collagen c-telopeptide (CTX) concentrations following 10 weeks of feeding trial diets. There were nine animals receiving FOS, eleven receiving HCMP –, HCMP+ and HCMPK +, and ten receiving HCMPK – (due to errors during analysis). Values with different superscripts (a) denote significant differences between groups at p<0.05.	84
Table 25: Pearsons correlation coefficients for <i>ex vivo</i> DEXA measurements on femurs of sixty rats, following 10 weeks of feeding trial diets. * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level.	85
Table 26: <i>Ex vivo</i> spine and femur area, bone mineral content (BMC) and densities (BMD) of rats, following 10 weeks of feeding trial diets. There were twelve animals per dietary group. Values with different superscripts (a) denote significant differences between groups in the same row at p<0.05.	87
Table 27: Correlation between rat weight, femur weight and length, and the biomechanical parameters measured after ten weeks of trial diets being consumed by sixty rats. * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level; ^{ns} indicates no significance.	88
Table 28: Confounders of biomechanical parameters, used as covariates during analysis of variance. Significance is determined using the Pearson’s Correlation coefficients in Table 27, and examination of scattergraphs in Figure 31 and Figure 32. NS indicates no significance, † indicates possible confounder.	91
Table 29: Results of biomechanical tests on right femurs following 10 weeks of feeding study diets. There were twelve rats per dietary group. Values with different superscripts (a,b) denote significant differences between groups in the same row at p<0.05.	92
Table 30: Pearson’s correlation coefficients for measurements of wet, dry and ashed femurs following 10 weeks of feeding trial diets to sixty rats. * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level; ns indicates no significance.	93
Table 31: Weight and composition of rat femurs following 10 weeks of feeding trial diets; there were twelve animals per dietary group. Values with different superscripts (a) denote significant differences between groups in the same row at p<0.05.	95
Table 32: Effect of diet on bone mineral content of rats fed trial diets for ten weeks; there were twelve animals per dietary group. Values with different superscripts (a) denote significant differences between groups in the same row at p<0.05.	96
Table 33: Effect of diet on ratio of mineral to ash in femurs of rats fed trial diets for ten weeks; there were twelve animals per dietary group. Values with different superscripts (a) denote significant differences between groups in the same row at p<0.05.	96
Table 34: Caecum counts of Colony Forming Units (CFUs) of animals fed different diets for ten weeks. There were twelve animals per dietary group. Values with different superscripts (a,b) denote significant differences between groups at p<0.05.	97

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 heritable 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.

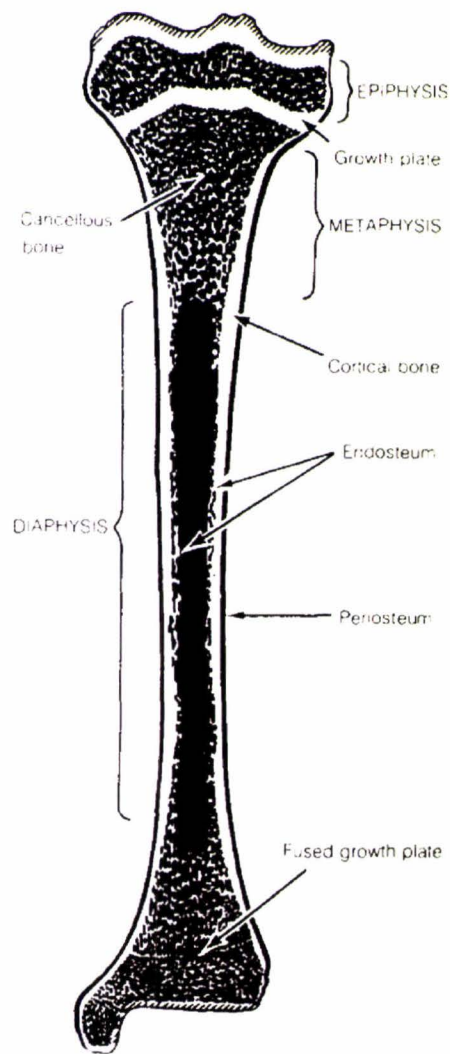


Figure 1: Anatomical features of a long bone, showing epiphysis, metaphysis and diaphysis. Taken from Baron (1999).

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.

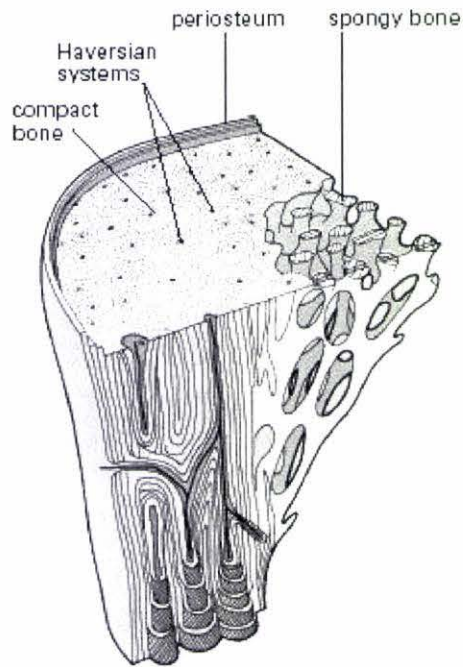


Figure 2: Cortical (compact) and trabecular (spongy) bone. Taken from Oxford University Press (2003).

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

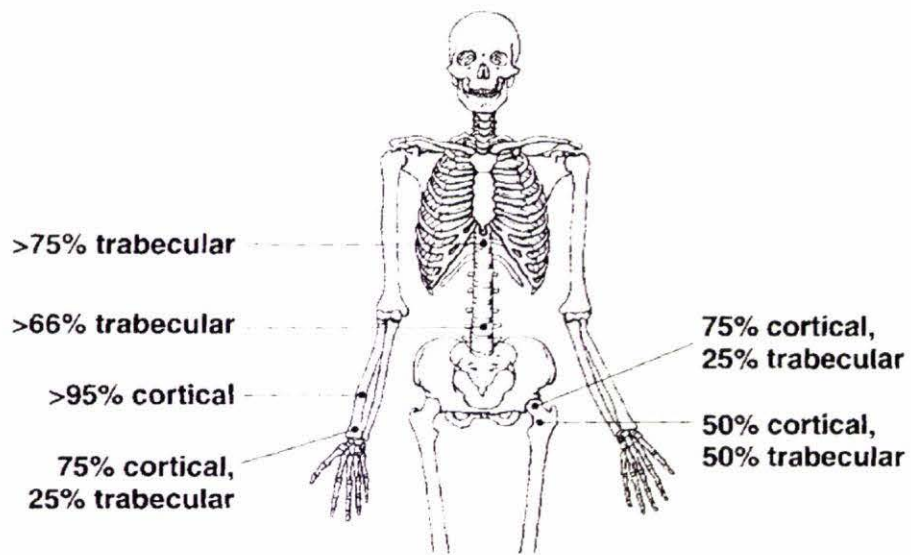


Figure 3: Relative distribution of cortical and trabecular bone in different parts of the skeleton. Taken from Mundy (1999).

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 ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). 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.

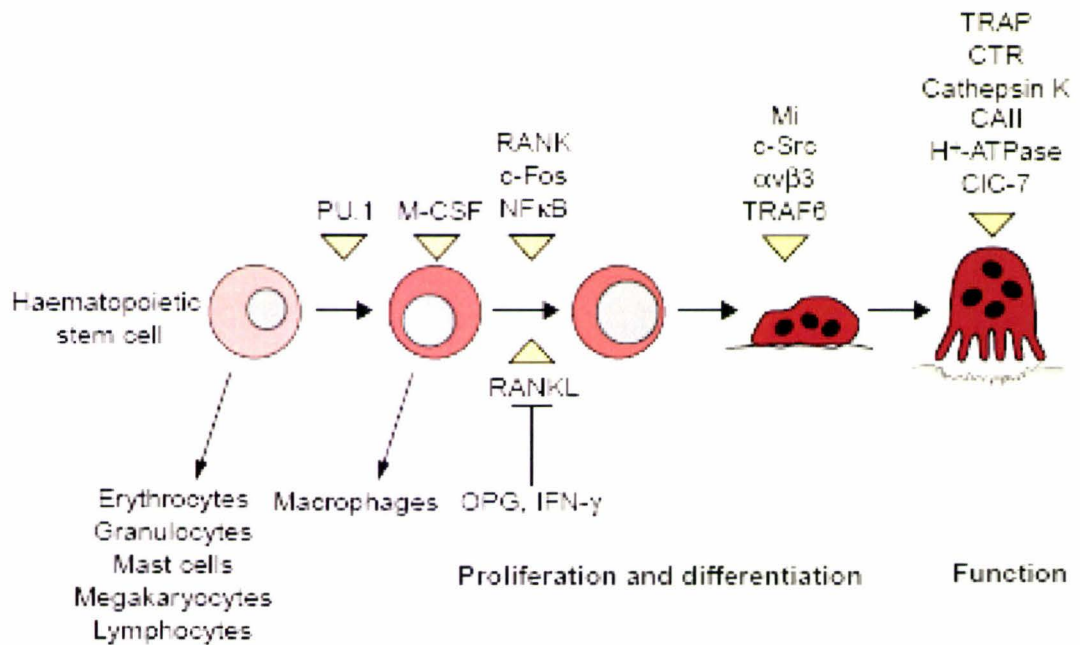


Figure 5: Signals required for osteoclast differentiation and function. $\alpha\beta3$, Vitronectin receptor; CAII, Carbonic anhydrase II; CTR, Calcitonin receptor; IFN- γ , Interferon- γ ; M-CSF, Macrophage colony-stimulating factor; Mi, Microphthalmia; OPG, Osteoprotogerin; RANK, Receptor activator of the NF- κ B; RANKL, Receptor activator of the NF- κ B 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- κ B 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- κ B (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- κ B, 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.

Osteoprotegerin (OPG; also known as osteoclastogenesis-inhibitory factor (OCIF), or TNF-receptor-like molecule 1 (TR1 or TNFr1), or follicular dendritic 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)-1 β , IL-6, IL-11, and TNF α 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.

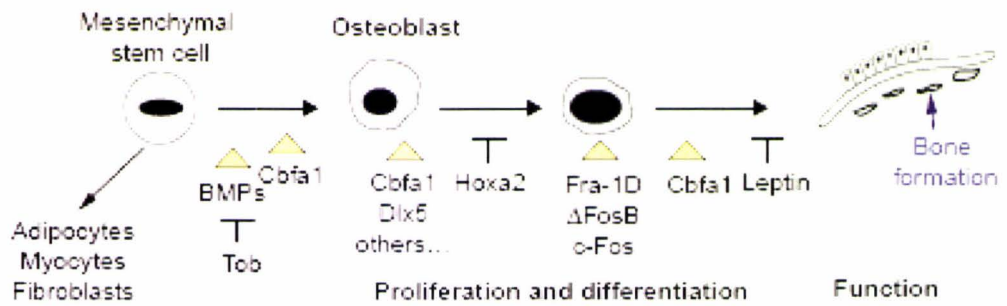


Figure 7: Signals required for osteoblast differentiation and function. Cuboidal osteoblasts are shown on the newly formed bone, together with some osteocytes embedded in the bone matrix. Adapted from Wagner and Karsenty (2001).

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).

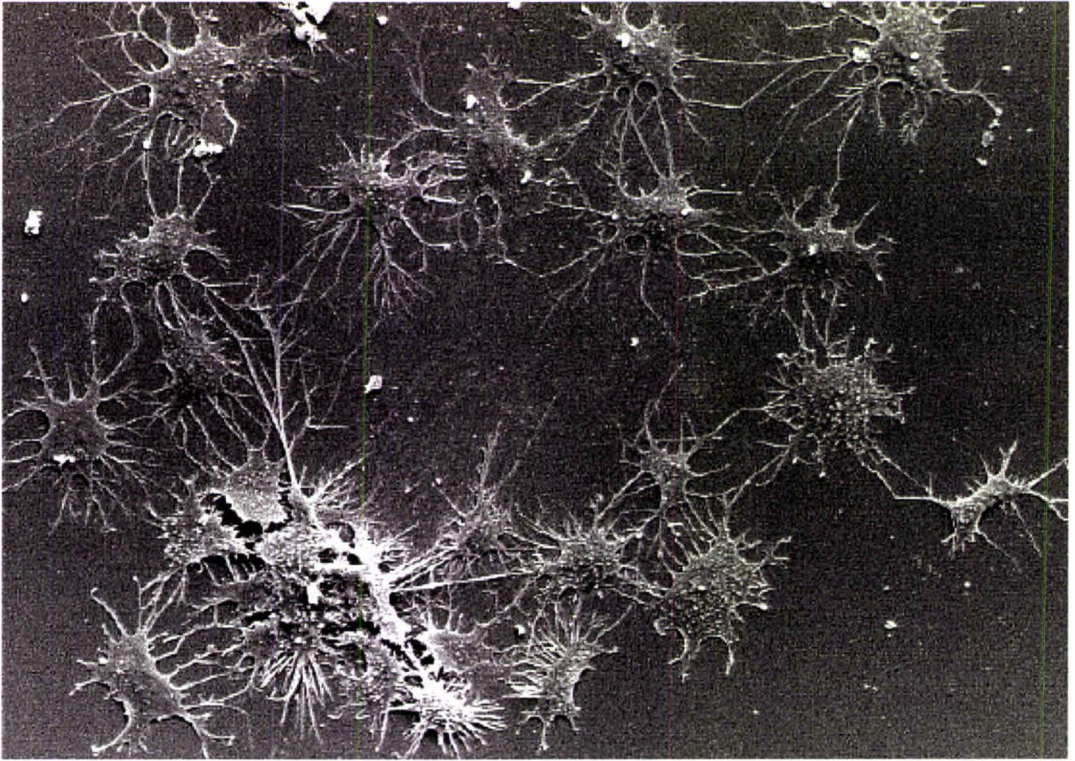


Figure 8: Scanning electron micrograph of osteocytes, isolated from embryonic chicken calvariae, following three days of culture as a monolayer. The lacunocanicular network can be seen between the osteocytes. Taken from Burger and Klein-Nulend (1999).

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 led 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.

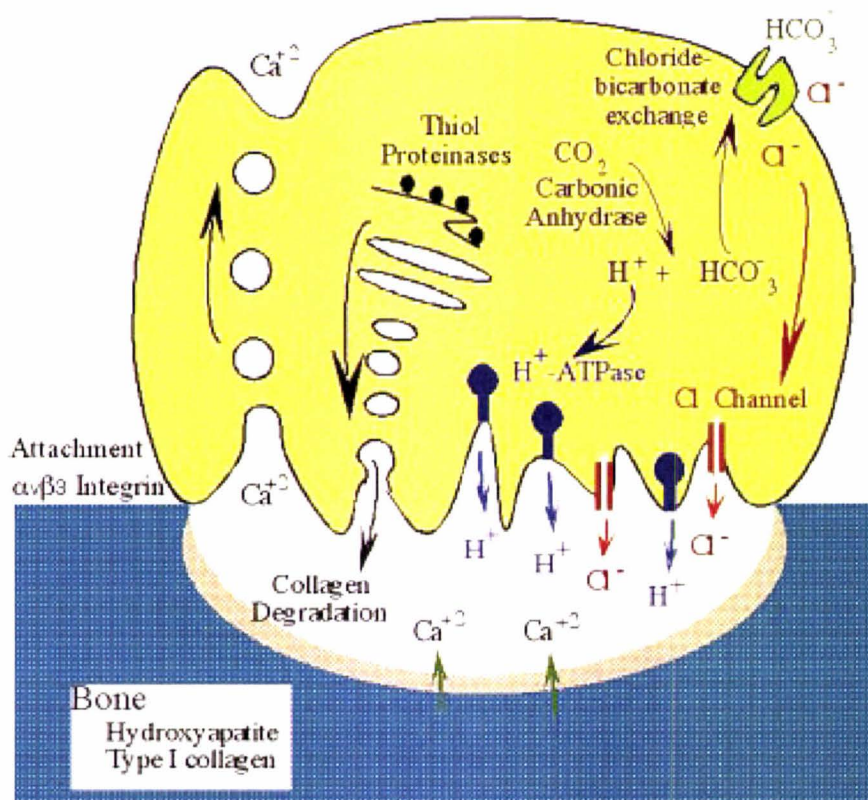


Figure 9: The osteoclast dissolving bone. Taken from Blair (2002).

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) $_2$ D (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 (Brandao 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. $1,25(\text{OH})_2\text{D}$ 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).

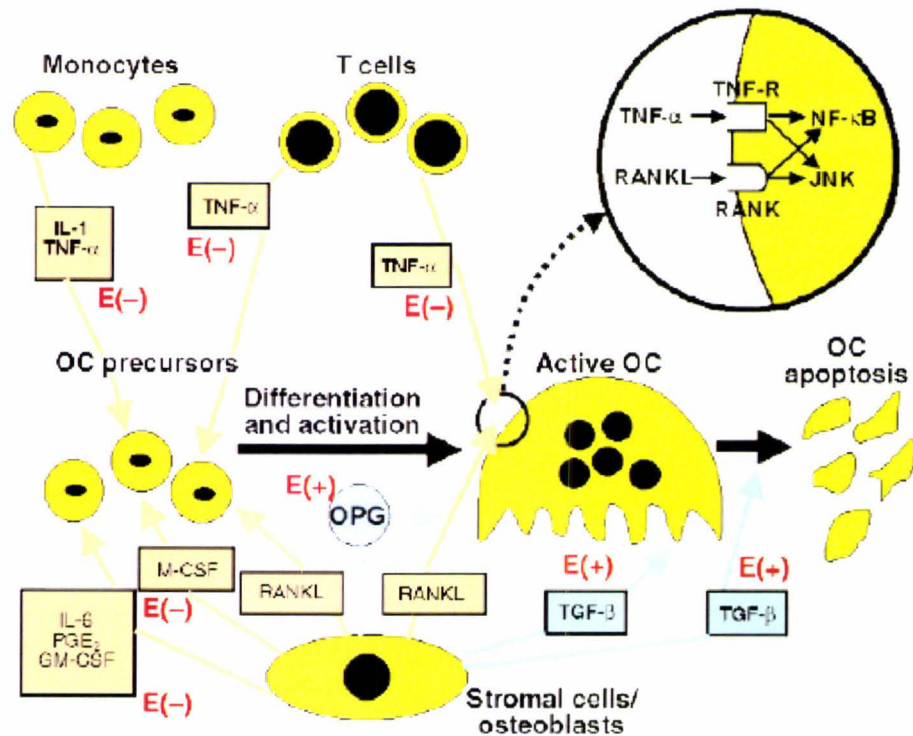


Figure 10: Actions of oestrogen (E) on cytokines in bone. Stimulatory (+) factors are shown in blue and inhibitory (-) effects are shown in orange. Taken from Riggs (2000).

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 (T₄) form (although some of the active Triiodothyroxine, T₃, is also produced). T₃ promotes long bone growth during development; in adults, excess can cause accelerated bone loss (Bassett and

Williams, 2003). T₃ 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-suppression 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 $1,25(\text{OH})_2\text{D}$ 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