

# **The Effect of Statins on Bone and Mineral Metabolism.**

*By*

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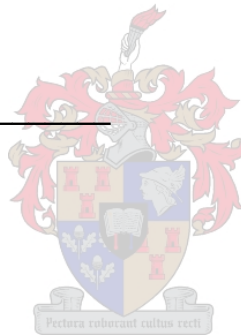
## Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not been previously, in its entirety or in part, been submitted at any University for a degree.

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**Frans Jacobus Maritz**

Date: \_\_\_\_\_



## Summary

### The Effect of Statins on Bone and Mineral Metabolism

Both statins and amino-bisphosphonates reduce the prenylation of proteins which are involved in cytoskeletal organization and activation of polarized and motile cells. Consequently statins have been postulated to affect bone metabolism. We investigated the effects of different doses of simvastatin (1,5,10 and 20mg/Kg/day), administered orally over 12 weeks to intact female Sprague-Dawley rats, and the effect of simvastatin 20mg/Kg/day in sham and ovariectomised rats, on femoral bone mineral density (BMD) and quantitative bone histomorphometry (QBH), compared to controls. Similarly, the effect of atorvastatin (2,5mg/Kg/day) and pravastatin (10mg/Kg/day) on BMD was investigated and compared to controls. BMD was decreased by simvastatin 1mg/Kg/day ( $p = 0.042$ ), atorvastatin ( $p = 0,0002$ ) and pravastatin ( $p = 0.002$ ). The effect on QBH parameters differed with different doses of simvastatin (ANOVA;  $p = 0.00012$ ). QBH parameters of both bone formation and resorption were equivalently and markedly increased by simvastatin 20mg/Kg/day in two independent groups of intact rats, and reflected by a relatively unchanged BMD. At lower doses, simvastatin 1mg/Kg/day decreased bone formation while increasing bone resorption as reflected by a marked decrease in BMD. Ovariectomised animals receiving simvastatin 20mg/Kg/day showed no change in BMD relative to the untreated ovariectomised controls, their increase in bone formation was smaller than in sham-operated rats receiving simvastatin and there was no change in bone resorption. The dose response curves of simvastatin for bone formation and resorption differed from each other.

From these studies it is concluded that:-

- a) low-dose simvastatin (1mg/Kg/day), atorvastatin 2.5mg/Kg/day) and pravastatin 10mg/Kg/day) decrease BMD in rodents;

- b) 1mg/Kg/day simvastatin decreases bone formation and increases bone resorption and is reflected by a reduced BMD;
- c) 20mg/Kg/day simvastatin increases bone formation and resorption and results in an unchanged BMD;
- d) the effects of simvastatin on QBH differ at different dosages;
- e) the dose-response curves for QBH parameters of bone resorption and bone formation differ from each other;
- f) the effects of simvastatin seen in intact rats are not observed in ovariectomised rats;
- g) simvastatin is unable to prevent the bone loss caused by ovariectomy.



## Opsomming

### Die Effek van Statiene op Been en Mineraal Metabolisme

Beide statiene en aminobisfosfonate verminder die prenelasie van proteïene wat betrokke is in die sitoskeletale organisasie en aktivering van gepolariseerde en beweeglike selle. Gevolglik is dit gepostuleer dat statiene 'n invloed sal hê op been metabolisme. Ons het die effekte van verskillende dossisse van simvastatien (1, 5, 10 en 20mg/Kg/dag), mondelings toegedien oor 12 weke aan intakte vroulike Sprague-Dawley rotte, en die effek van simvastatien 20mg/Kg/dag op skyn- en ge-ovariektoemeeerde rotte, op femorale been mineral digtheid (BMD) en kwantitatiewe been histomorfometrie (KBH), vergeleke met kontroles, ondersoek. Op 'n soortgelyke manier is die effek van atorvastatien (2,5mg/Kg/day) en pravastatien (10mg/Kg/dag) op BMD ondersoek en vergelyk met kontroles. BMD is verminder deur simvastatien 1mg/Kg/dag ( $p = 0.042$ ), atorvastatien ( $p = 0.0002$ ) en pravastatien ( $p = 0.002$ ). Die effekte op KBH parameters het verskil met verskillende dossisse van simvastatien (ANOVA;  $p = 0.00012$ ). KBH parameters van beide been vormasie en resorpsie is vergelykend en merkbaar verhoog deur simvastatien 20mg/Kg/dag in twee onafhanklike groepe van intakte rotte en is vergesel deur 'n relatiewe onveranderde BMD. Met laer dossisse het simvastatien 1mg/Kg/dag been vormasie verminder terwyl been resorpsie verhoog is en is weerspieël deur 'n merkbaar verminderde BMD. Ge-ovariektoemeeerde diere wat simvastatien 20mg/Kg/dag ontvang het, het geen verandering in BMD relatief tot die onbehandelde ge-ovariektoemeeerde kontroles getoon nie, en die toename in been vormasie was kleiner as in die skyngeopereerde rotte wat simvastatien ontvang het en daar was geen verandering in been resorpsie nie. Die dosis-respons kurwes vir simvastatien vir been vormasie en resorpsie het van mekaar verskil.

Uit hierdie studies word die volgende gevolgtrekkings gemaak:-

- a) lae-dosis simvastatien (1mg/Kg/dag), atorvastatien 2.5mg/Kg/dag en pravastatien 10mg/Kg/dag verminder BMD in knaagdiere;
- b) 1mg/Kg/dag simvastatien verminder been vormasie en verhoog been resorpsie en veroorsaak gevolglik 'n velaging in die BMD;
- c) 20mg/Kg/dag simvastatien verhoog been vormasie en resorpsie met 'n gevolglike onveranderde BMD;
- d) die effekte van simvastatien op KBH verskil met verskillende dossisse;
- e) die dosis-repons kurwes van been resorpsie en been vormasie verskil van mekaar
- f) die effekte van simvastatien wat waargeneem in intakte rotte word nie gesien in ge-ovariëktomeerde rotte nie;
- g) simvastatien kan die verlies van been wat veroorsaak word deur ovariektomie voorkom nie.

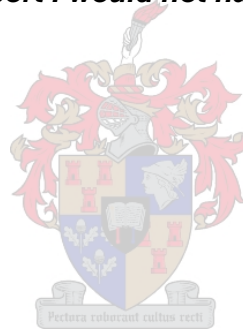


## Dedication

To

*Cheryl, David and Mark.*

*Without your support I would not have seen the end of this work.*



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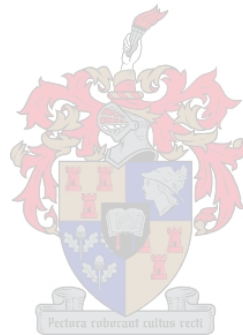


## Publications

Parts of this thesis have been published as follows:-

1. Maritz FJ, Conradie MM, Hulley P, Hough FS. Statins increase quantitative histomorphometric parameters of bone formation and resorption, and decrease bone density in rodents. *Arterio Thromb Vasc Biol* 2001; 21: 1636-1641.
2. Maritz FJ, Conradie MM, Gopal R, Hulley P, Hough FS. Statins increase bone formation and resorption, and decrease bone density in rodents. *Journal of Endocrinology Metabolism and Diabetes of South Africa* 2001; 6: 26-26. Abstract.
3. Maritz FJ, Conradie MM, Hulley P, Hough FS. The influence of an HMG-CoA reductase inhibitor on rat bones after ovariectomy. *S Afr Med J* 1999; 89: 478-478. Abstract.
4. Maritz FJ, Conradie MM, Hulley P, Hough FS. A comparison of the effect of equivalent doses of simvastatin, atorvastatin and pravastatin on bone mineral density in rodents. *Journal of Endocrinology Metabolism and Diabetes of South Africa* 2000a; 5: 39-39. Abstract.
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6. Maritz FJ, Conradie MM, Hulley P, Hough FS. Statins increase bone formation and resorption, and decrease bone mineral density in rodents. *Journal of Endocrinology Metabolism and Diabetes of South Africa* 2000c; 5: 47-47. Abstract.

7. Maritz FJ, Conradie MM, Hulley P, Hough FS. The effect of statins on bone mineral density and quantitative bone histomorphometry in rodents. *Osteoporosis International* 2002; 13 (Suppl.): S13. Abstract.



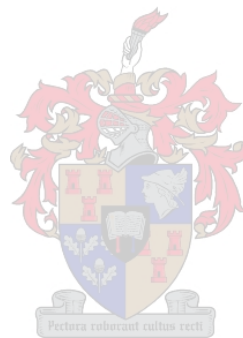
## Congress Proceedings

Parts of this thesis have been presented at Local, National and International Scientific Meetings:

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4. Maritz FJ, Conradie MM, Hulley P, Hough FS. Simvastatin increases bone formation and resorption in rodents. 36<sup>th</sup> LASSA Congress. Durban, 2-7 April 2000. Oral presentation.
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6. Maritz FJ, Conradie MM, Hulley P, Hough FS. Statins increase bone formation and resorption, and decrease bone mineral density in rodents. 36<sup>th</sup> SEMDSA Congress. Durban, 2-7 April 2000. Oral presentation.
7. Maritz FJ, Conradie MM, Gopal R, Hulley P, Hough FS. The effects of simvastatin and pravastatin on bone mineral density and quantitative bone histomorphometry. 44<sup>th</sup> Annual Academic Day, University of Stellenbosch Medical School, Tygerberg, August 2000. Oral presentation.
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## List of abbreviations

In addition to the conventional atomic symbols and S. I. Units, the following abbreviations are used in this thesis:

ADP:	Adenine Diphosphate
ANOVA:	Analysis of Variance
A:	Atorvastatin
BMD:	Bone Mineral Density
C:	Control
FTase:	Farnesol transferase
QBH:	Quantitative Bone Histomorphometry
KBH:	Kwantitatiewe Been Histomorfometrie
GAP:	GTPase Activating Proteins
GDP:	Guanine Dinucleotide Phosphate
GDI:	GDP Dissociation Inhibitor
GEF:	GTP Exchange Factors
GGTase I:	Geranylgeraniol transferase type I
GGTase II:	Geranylgeraniol transferase type II
GTP:	Guanine Trinucleotide Phosphate
GTPase:	Guanine Trinucleotide Phosphatase
HMG-Co:	Hydroxymethylglutaryl Coenzyme-A
LDL:	Low Density Lipoprotein
LASSA:	Lipid and Atherosclerosis Society of Southern Africa
NO:	Nitric Oxide
NOS:	Nitric oxide synthase
OVX:	Ovariectomy
OVX-S:	Ovariectomy plus Statin
PI3 kinase:	Phosphatidylinositol-3-phosphate



PP: Pyrophosphate

SEMDSA: Society for Endocrinology, Metabolism and Diabetes of South Africa

Sh: Sham

Sh-S: Sham plus Statin

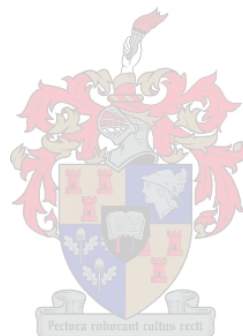
S20: Simvastatin 20mg/Kg/day

S10: Simvastatin 10mg/Kg/day

S5: Simvastatin 5mg/Kg/day

S1: Simvastatin 1mg/Kg/day

VLDL: Very Light Density Lipoprotein



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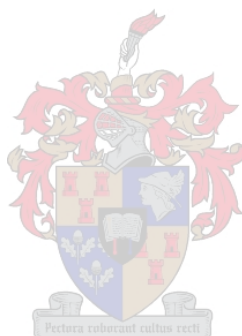
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## **Chapter 1: Background and Literature review.**

### **1.1. Introduction.**

Osteoporosis affects a sizable proportion of Westernised societies, particularly females. The lifetime risk of a fracture in Caucasian women is thought to be in the region of 30 – 40%. [1993] Accurate figures for South Africa are hard to come by. It is estimated that the incidence of osteoporosis in the White, Asian and Coloured (peoples with an ethnic admixture) populations is similar to that of Caucasians in developed countries, whereas the disease is less common in the South African Black populations. [Daniels ED, Pettifor JM et al., 1997] The incidence of osteoporosis increases with advancing age in a similar fashion to cardiovascular disease and it is not uncommon to find these two conditions occurring together. [Solomon L, 1979]

Cardiovascular diseases, including coronary artery disease and strokes, are the leading causes of mortality and morbidity in the United States of America (USA) followed by lung and colon cancer, diabetes and chronic obstructive pulmonary disease. [Doyle R, 2001] The incidence of coronary artery disease and associated risk factors, including dyslipidaemia, are similarly high in the South African White, Asian and Coloured ethnic groups, exceeding the prevalence of most Westernised societies in Europe and the North Americas. [Steyn K, Jooste PL et al., 1985] Co-incidentally, the prevalence of coronary artery disease, and the associated dyslipidaemia, is much lower in the South African Black peoples than in the other ethnic groups. [Steyn K, Jooste PL et al., 1991] There is anecdotal evidence that these figures on the incidence of coronary artery disease in South African Blacks may be on the rise due to the adoption of a Westernised lifestyle. However, there are no data to support this supposition and indeed, there is evidence in favour of the contrary. [Walker AR, Adam A, and Küstner HG, 1993] Nonetheless, atherosclerosis and strokes are not uncommon in the Black populations despite the relatively low incidence of dyslipidaemia. [Fourie J and Steyn K, 1995]



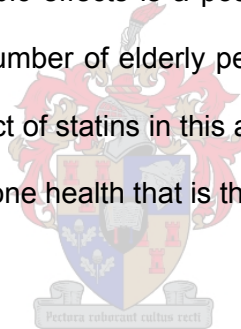
The associated risk factors for atherosclerosis are increasingly being targeted for aggressive management, and dyslipidaemia has found itself most amenable to this attack. [Nass CM, Wiviott SD et al., 2000] The advent of the newer and highly effective lipid-lowering agents such as the hydroxymethylglutaryl-CoA (HMG-CoA) Reductase Inhibitors (statins), has introduced a potent tool for the reduction of cholesterol which effectively reduces the risk of cardiac events. [Farnier M and Davignon J, 1998; Farnier M, 1999] The increasingly lenient and broadened guidelines for the use of statins has meant that more people with, or at risk of, osteoporosis are exposed to these agents. Indeed, the statins are among the most commonly used drugs, with more than 3 million Americans taking a statin every day. [Gotto AMJ, 1997; Mundy GR, 2001]

The statins are potent lipid-lowering agents that inhibit the rate-limiting enzyme of the cholesterol synthetic pathway, namely HMG-CoA reductase. [Farnier M and Davignon J, 1998] Consequently they reduce the intracellular free cholesterol pool. The reduction of this cholesterol pool may, with the more potent and longer acting statins, reduce lipoprotein production by the liver and especially the production of the very low-density lipoproteins (VLDL). [Farnier M and Davignon J, 1998; Mundy GR, 2001; Stein EA, Lane M, and Laskarzewski P, 1998] However, this is not the primary mode of action by which they lower serum low-density lipoprotein (LDL)-cholesterol. By reducing the intracellular cholesterol pool, the statins induce the synthesis of LDL-receptor protein and increase the cell surface expression of these receptors. This consequently leads to an increased uptake of LDL from the serum, which in turn reduces the serum LDL-cholesterol concentration.

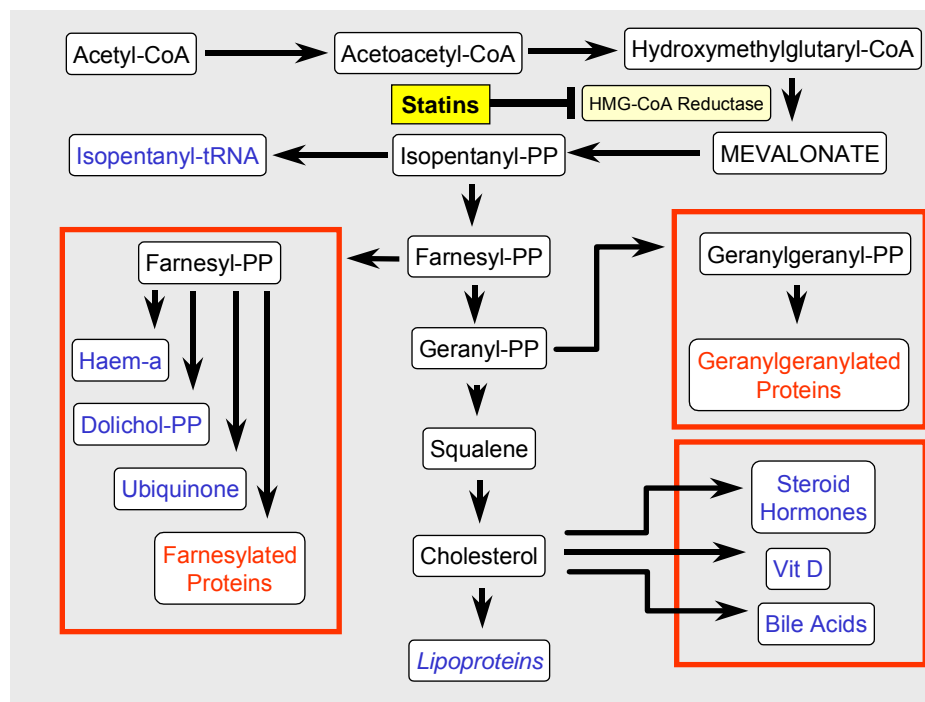
The statins have different pharmacokinetic properties based on their lipid solubility and metabolism. [Beaird SL, 2000; Corsini A, Bellosta S et al., 1999b] In addition they differ in their duration of action and their potency. [Dansette PM, Jaoen M, and Pons C, 2000; Corsini A, Bellosta S et al., 1999b; Wolffenbuttel BH, Mahla G et al., 1998] The statins have been classified into the synthetic and the natural statins, according to which

they supposedly have effects on conventional non-lipid cardiovascular risk factors that distinguish them from each other. [Mundy GR, 2001; Rosenson RS and Tangney CC, 1998] In addition the statins have been found to have other non-lipid-lowering effects which may reduce cardiovascular risk. Amongst these are antithrombotic, vasodilative, antioxidant, anti-inflammatory and anti-proliferative effects that may participate in stabilisation of the endothelium. Other organs systems may also be involved in these mechanisms. [Bellosta S, Bernini F et al., 1998; Corsini A, Bellosta S et al., 1999a; Laufs U and Liao JK, 2000; Farnier M and Davignon J, 1998; Mundy GR, 2001; Wheeler DC, 1998] These non-lipid-lowering effects are referred to as the pleiotropic effects of the statins.

Included in these pleiotropic effects is a postulated effect of statins on bone and mineral metabolism. Given the number of elderly persons who are taking statins it would be important to delineate the effect of statins in this age group that is particularly at risk for osteoporosis. It is this effect on bone health that is the theme of this thesis.



## 1.2. The mevalonate and cholesterol synthetic pathway and protein prenylation.

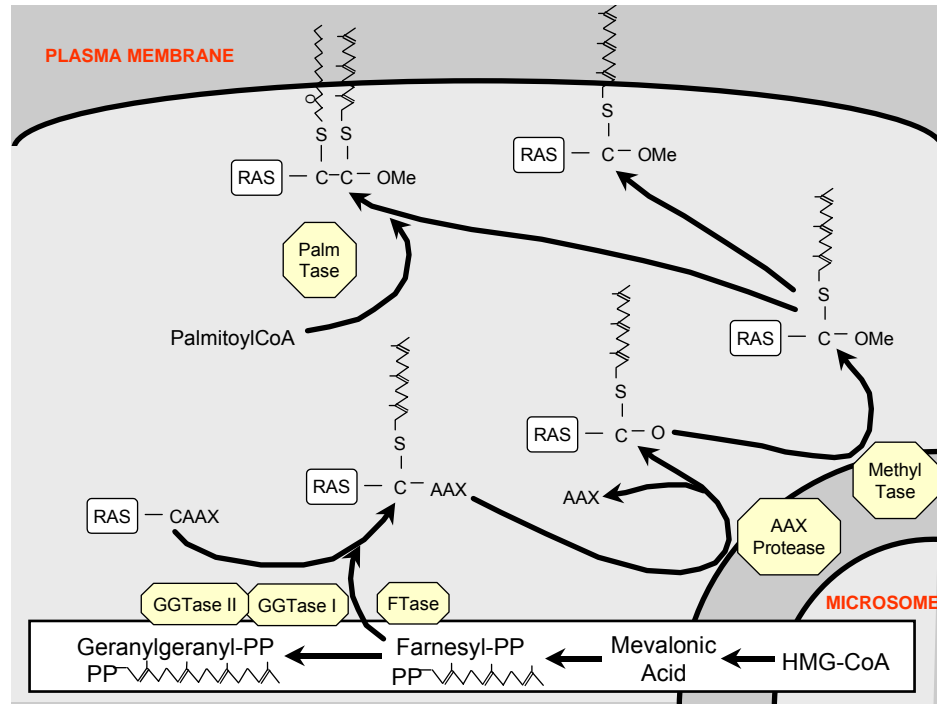


**Figure 1.1. The mevalonate/cholesterol synthetic metabolic pathway.**

Important products of this pathway include the prenylated proteins – the farnesylated and geranylgeranylated proteins to which farnesylpyrophosphate and geranylgeranylpyrophosphate have been added.

Cholesterol and other sterols such as steroid hormones, bile salts and vitamin D are widely known derivatives of the mevalonate metabolic pathway (Fig. 1.1). There are however, less well known products of this pathway that have important physiological roles; dolichol in glycoprotein biosynthesis; the side chain of ubiquinone, an important component of the mitochondrial electron transport chain; isopentanyl adenosine, a component of isopentanyl transfer-RNA; the farnesylpyrophosphate side chain of haem-a, the iron-binding nucleus of haemoglobin; and the important and only relatively recently discovered prenylated proteins. It has also become evident that other intermediates of the cholesterol synthetic pathway play an important role in signal transduction and other cellular processes. Farnesylpyrophosphate and geranylgeranylpyrophosphate are added to the carboxy-terminal of numerous cytosolic proteins to form prenylated proteins, which

have diverse cellular functions (Fig. 1.2). The discovery of these prenylated proteins has provided many new insights into cellular biology and opened up novel therapeutic possibilities.



**Figure 1.2. The prenylation of proteins.**

Farnesylpyrophosphate or geranylgeranylpyrophosphate are added by one of three prenyl-transferases, followed by removal of the three terminal amino acids, and the addition of a methyl and palmitoyl molecule. Abbreviations: GGTase I = geranylgeraniol transferase type I; GGTase II = geranylgeraniol transferase type II; FTase = farnesol transferase; Methyl Tase = methyl transferase; Pal Tase = palmitoyl transferase.

It became evident early on that the inhibition of mevalonate synthesis by the statins, and the subsequent depletion of the endogenous mevalonate pool, resulted in a cessation of cell cycling and DNA synthesis that is associated with pronounced changes in cell morphology. Even suppression of tumor growth was noted. [Brown MS and Goldstein JL, 1980] These changes could be reversed by supplying exogenous mevalonate to the arrested cells or by removing the inhibitor. This restoration of cell growth and morphology could not be reproduced by adding cholesterol, dolichol,

ubiquinone or isopentanyl adenosine, suggesting that some other metabolite of mevalonate was responsible for these changes. Subsequently it was demonstrated that when radiolabeled mevalonate was added to the medium, radioactivity was incorporated into a wide range of cytosolic and membrane-bound proteins. This occurred via the covalent attachment of the isoprene products of mevalonate, farnesol and geranylgeraniol, to these proteins, a process thereafter referred to as prenylation, and the modified proteins as prenylated proteins. [Maltese WA, 1990]

The proteins destined to be prenylated are characterised by a carboxy-terminal CAAX box of amino acids where C represents cysteine, A an aliphatic amino acid and X any amino acid (Fig. 1.2). These terminal amino acid motifs, and in some cases certain additional upstream sequences, act as recognition sites for prenyl transferase enzymes. [Moore SL, Schaber MD et al., 1991] The prenyl transferase attaches the respective prenyl group, farnesylpyrophosphate or geranylgeranylpyrophosphate, to a carboxy-terminal cysteine of the protein. At least 3 prenyl transferases are known to exist and have been characterised. Farnesol transferase (FTase) and geranylgeraniol transferase I (GGTase I) recognise a CAAX box and the terminal X of the CAAX box determines whether farnesol or geranylgeraniol is added to the protein. Geranylgeraniol transferase II (GGTase II) recognises CC, CXC and CCXX motifs and is active on a distinct group of Rab proteins. [Zhang FL and Casey PJ, 1996] FTase and GGTase I are heterodimeric enzymes which share a common  $\alpha$ -subunit that binds to the relevant prenyl group. They have different but homologous  $\beta$ -subunits, which recognise the different CAAX sequences of the target protein. GGTase II is somewhat different and has two subunits analogous to the other transferases but with an additional third subunit required for enzymatic activity. These differences from the other prenyl transferases may have therapeutic implications particularly for bone metabolism. A bisphosphonate which specifically inhibits this enzyme has been developed. [Coxon FP, Helfrich MH et al., 2001; Coxon FP, Dunford JE et al.,

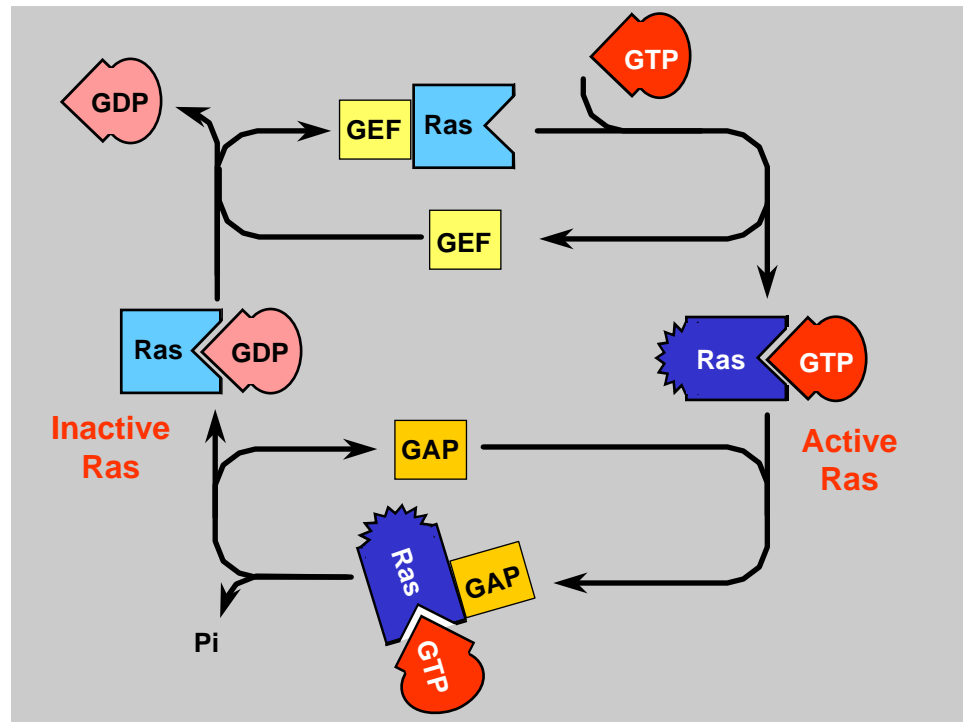
2001] This is but one example of a drug that interferes with the cholesterol synthetic pathway and is also used to manipulate bone metabolism.

Prenylation is the first of 3 sequential steps that render these prenylated proteins active (Fig. 1.2). These modifications primarily confer lipid solubility and consequently membrane binding to the prenylated protein. Prenylation is followed by the proteolytic cleavage of the terminal 3 amino acids by a microsomal carboxypeptidase, which is then followed by the addition of a methyl group to the remaining terminal cysteine by a microsomal aminotransferase. Some prenylated proteins undergo further modification by the addition of a palmitoyl molecule to a more proximal cysteine. [Hancock JF, Magee AI et al., 1989]

In all cases prenylation is essential for the activity of all these proteins. If the terminal CAAX box is removed or blocked, if the relevant prenyl transferase is inhibited, or if the availability of the prenyl substrate is diminished as is found with the inhibition of the cholesterol synthetic pathway by statins, then these proteins are inactive. [Kato K, Cox AD et al., 1992] The additional modifications of amino acid cleavage and methylation are also required, and sometimes essential, but mostly serve to complement prenylation in the activation of these proteins. [Zhang FL and Casey PJ, 1996] Although the bulk of the prenylated proteins are cytosolic in location, they are active only in their membrane bound form and both prenylation and palmitoylation render these proteins lipid soluble thus allowing them to bind to membranes. In addition to their role in membrane binding these post translational modifications are also important for interactions with other regulatory proteins of the small GTP-binding proteins. [Cox AD and Der CJ, 1992]

The prenylated proteins have diverse functions and include the nuclear lamins, the  $\gamma$ -subunit of the heterotrimeric receptor-associated G proteins, various retinal proteins and by far the largest group, the family of Ras-related small GTP-binding proteins that play an essential role in the normal function of cells. [Cox AD and Der CJ, 1992]

### 1.3. Small GTP-binding proteins

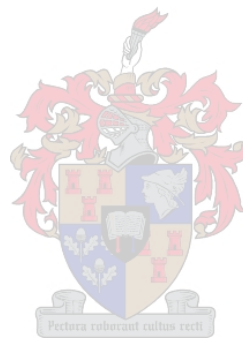


**Figure 1.3. The Ras related GTP-binding proteins act as molecular switches.**

This scheme applies to all the other small Ras-related GTP-binding proteins as well as the heterotrimeric receptor-associated G proteins. These proteins are only active in their GTP-bound membrane-associated form, which is modulated by other regulatory proteins. Active GTP-bound Ras has an intrinsic GTPase activity that is further enhanced by GTPase Activating Proteins (GAP) resulting in the formation of GDP-bound inactive Ras. The subsequent exchange of GDP for GTP is regulated by GTP Exchange Factors (GEF) (also known by other names such as GDP Dissociation Inhibitor GDI). These GEFs (or GDI's) generally inhibit the exchange of GDP for GTP but also cover the prenylation site on Ras making it less lipid soluble and unbinding it from the membrane, with the result that inactive GDP-bound Ras is cytosolic in position. With the removal of GEF (or GDI), the prenylation site is uncovered, GDP is exchanged for GTP and the active GTP-bound Ras becomes membrane bound at its active site. Defects in this switching mechanism gives rise to disease. Some mutations of Ras lack intrinsic GTPase activity and are consequently continuously active, a situation seen in numerous common cancers. [Takai Y, Kaibuchi K et al., 1993]

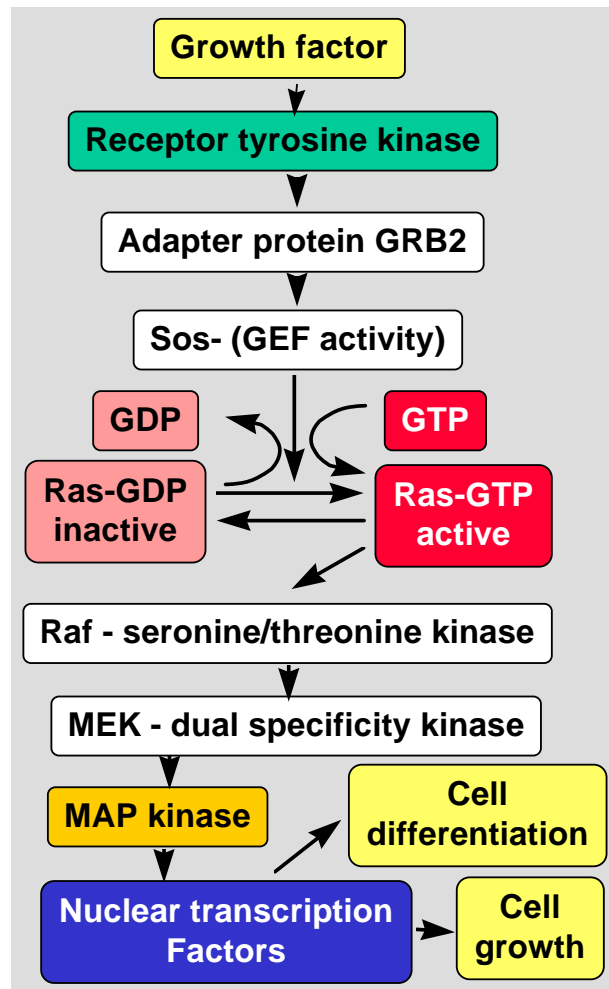
The small GTP-binding proteins comprise a large super-family of Ras-related proteins of which the **Ras**, **Rab**, **Rho**, and **Rac**, families are amongst those which are

prenylated. Prenylation serves to make these proteins more lipid-soluble and able to bind to the lipid cell membranes. These proteins cycle between the active GTP-bound and the inactive GDP-bound forms (Fig. 1.3). This cycle is modulated by their interaction with a large group of regulatory proteins. This interaction with the regulatory proteins is further influenced by the prenylation state of the small GTP-binding proteins. [Bokoch GM and Der CJ, 1993]





*The Ras family of small GTP-binding proteins*



**Figure 1.4. Ras proteins in signal transduction.**  
 Ras is a pivotal link between Tyrosine Kinase Receptors and the activation of nuclear transcription factors leading to, amongst other activities, cell differentiation and growth. It is via this pathway that constitutionally active forms of Ras result in cancer. Without prenylation Ras cannot participate in this pathway.

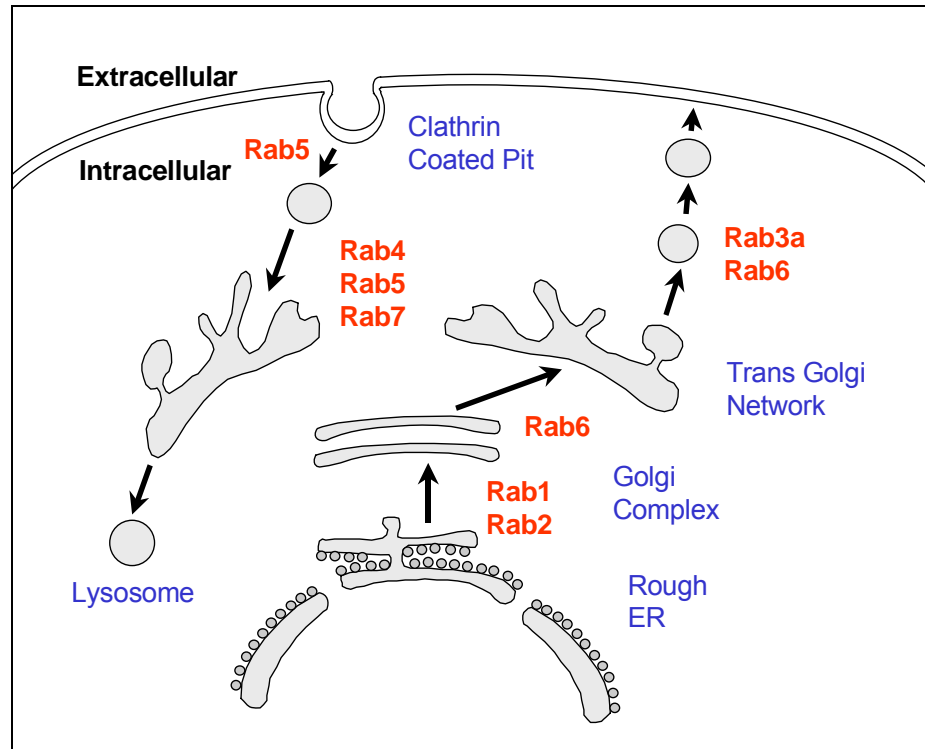
The Ras family of small GTP-binding proteins acts as an important component of the cell's signal transduction pathway between tyrosine kinase receptors on the one hand and the cell nucleus and other effectors on the other hand, leading to, amongst others,

cell growth, cell differentiation and metabolic processes (Fig. 1.4). Unlike the other members of the small GTP-binding family of proteins, which are geranylgeranylated, the Ras proteins are farnesylated. The function of Ras is critically dependent on its prenylation state and without farnesylation these Ras proteins are inactive and cannot perform their function. [Kato K, Cox AD et al., 1992] Certain mutant and oncogenic forms of Ras lack intrinsic GTPase activity and are consequently unable to switch to the inactive GDP-bound form. They are therefore constitutively active and are associated with, and lead to, the formation of a variety of human cancers. [Rao KN, 1995] When the prenylation of these oncogenic Ras mutations is prevented, including via the use of statins, they lose their oncogenic capacity. [Kawata S, Nagase T et al., 1994] The realisation that prenylation plays a pivotal role in cell growth and differentiation raised the possibility that prenylation might play a role in carcinogenesis [Rao KN, 1995] and that inhibition of this process could have therapeutic possibilities. [Gibbs JB and Oliff A, 1997] Inhibitors of prenylation have since been used as important adjuvants to cancer chemotherapy. [Lerner EC, Hamilton AD, and Sebti SM, 1997; Mundy GR, 1997]

Statins inhibit the cholesterol synthetic pathway and thereby reduce the availability of the substrates for prenylation, namely farnesylpyrophosphate and geranylgeranylpyrophosphate. Via their reduction of prenyl group availability, and consequently via their inhibition of prenylation, it is supposed that statins might have effects other than just the reduction of plasma LDL-cholesterol. These effects include an inhibition of cell growth and differentiation possibly via an inhibition of Ras. [Bellosta S, Ferri N et al., 2000b; Kawata S, Nagase T et al., 1994] Cross-sectional studies initially suggested an association between low cholesterol levels and malignancy, and there was a concern that statins might promote cancer. However, it was subsequently found that persons who already had a malignancy or other advanced disease at the time of the observations caused these observed low serum cholesterol levels. It is reassuring to note that users of statins are less likely to develop a cancer and this observation may well be

related to the effects that statins have on prenylation. [Blais L, Desgagne A, and LeLorier J, 2000]

*The Rab family of small GTP-binding proteins*

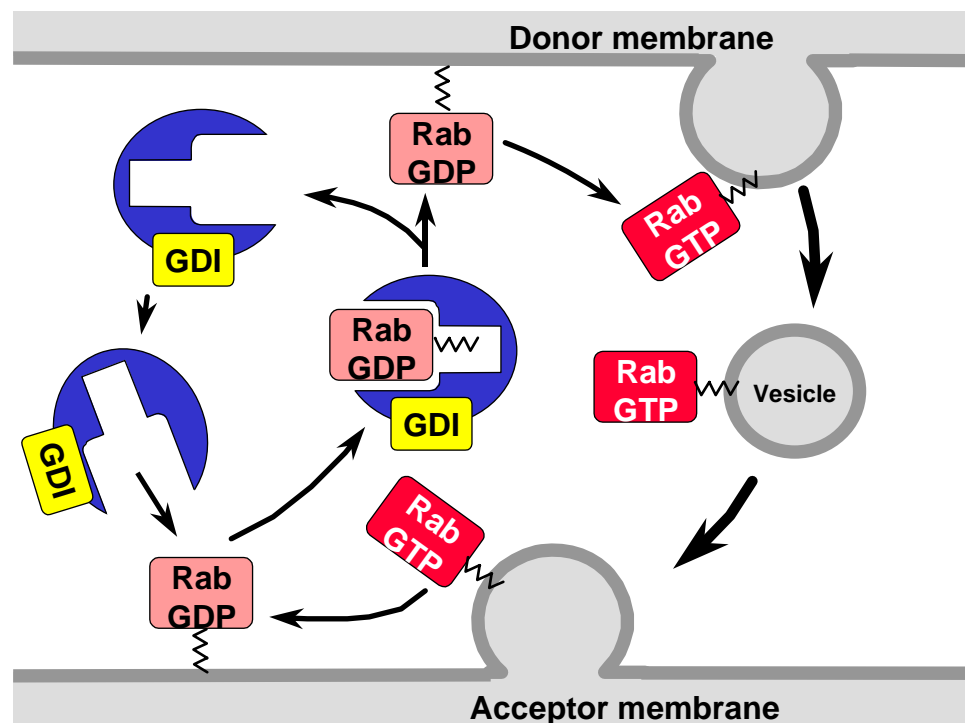


**Figure 1.5. The Rab proteins.**

These are members of the small GTP-binding proteins and play an important role in endocytosis, exocytosis and trafficking of vesicles between different compartments. This is crucial not only for the function of the endocrine pancreas and other endocrine organs but also for most other cells including osteoclasts.

Further targets of prenylation inhibiting drugs are the Rab proteins. The Rab family of small GTP-binding proteins is intimately involved in the regulation of intracellular vesicular transport, exocytosis and endocytosis, as well as targeting of vesicles between different organelles and the cell surface membrane (Fig. 1.5). [Kinsella BT and Maltese WA, 1991] It is therefore to be expected that the Rab proteins will play an important role in all cells, but particularly in those involved with the cycling of intracellular organelles. The

isoprenylation of these Rab proteins is critical for their association with specific intracellular compartments and regulation of vesicular transport processes. Prenylation also plays an important role by modulating the interaction between Rab and the regulatory proteins that determine their ATP or ADP binding, and consequently membrane binding. [Takai Y, Kaibuchi K et al., 1993] GDP Dissociation Inhibitor (GDI) is one such regulatory protein, which regulates the GDP and GTP binding of Rab and helps to shuttle Rab between donor and acceptor membranes (Fig. 1.6). [Alexandrov K, Horiuchi H et al., 1994]



**Figure 1.6. The interaction between Rab and GDI proteins.**  
 These proteins help to shuttle organelles between donor and acceptor membranes.

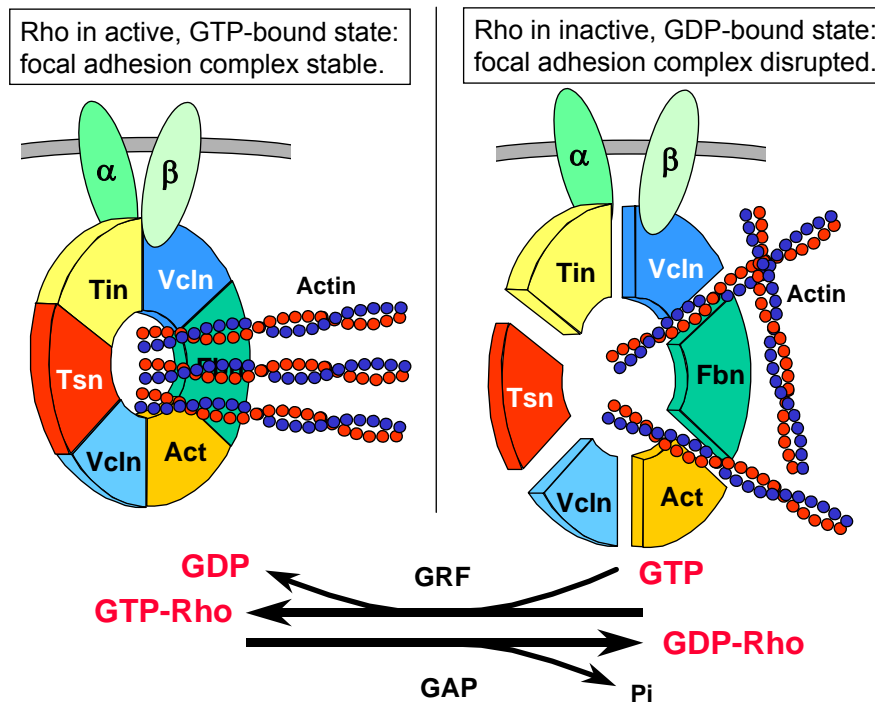
The Rab family is geranylgeranylated by GGTase II. The geranylgeranylation of these proteins therefore means that, experimentally, the effects of prenylation inhibitors on these Rab proteins can be expected to be reversed by the addition of geranylgeranylpyrophosphate instead of farnesylpyrophosphate. GGTase II is also

somewhat different from the other prenyl transferases in that it recognises carboxy-terminal sequences other than the CAAX. This raises the possibility that there may be a large family of these transferases. Furthermore, GGTase II requires another protein for activity, namely Rab Exchange Protein (REP). REP is homologous to GDI and is required in all cells. [Alexandrov K, Horiuchi H et al., 1994] A mutation of this protein was found to be responsible for choroideremia, an inherited X-linked disease that results in a slow degeneration of the retina ultimately leading to blindness. There are no other systemic features in this disease suggesting that there might be other isoforms of REP. [Cremers FP, Armstrong SA et al., 1994] A further search has led to the discovery of a closely related protein which is active in cells other than the retina, now named REP2, and the retinal protein REP1. [Zhang FL and Casey PJ, 1996]

Extensive intracellular vesicular trafficking is essential for the polarisation and bone resorbing activities of osteoclasts and it is to be expected that the Rab proteins will play an important role in the function of these cells. Rab 3 isoforms are expressed in bone marrow macrophages and their expression is increased by cytokines that promote the osteoclastic differentiation of these cells. Of note is that the Rab-3 co-localises with the H<sup>+</sup>ATPase or the vacuolar proton pump of osteoclasts. [Abu-Amer Y, Teitelbaum SL et al., 1999]

It is clear that Rab proteins play an important role in osteoclast function. Their inhibition might be an important method by which certain drugs exert their antiresorptive properties.

*The Rho family of small GTP-binding proteins.*



**Figure 1.7. The Rho proteins.**

Rho members of the small GTP-binding proteins play a pivotal role in the cytoskeleton via focal adhesion complex and stress fibre assembly. This explains the morphological changes observed when statins are added to cell cultures and which can be reversed by the addition of mevalonate.

Abbreviations: Tsn = tensin; Vcln = vinculin; Tin = talin; Fbn = fibrinin; Act = actin.

The Rho family of small GTPase proteins, comprising Rho, Rac and CDC42, plays a central role in the cytoskeletal organisation of polymerised actin (Fig. 1.7). [Craig SW and Johnson RP, 1996]. These changes are pivotal to the activation and function of motile and polarised cells such as macrophages and osteoclasts.

Rho is geranylgeranylated by GGTase I. However, under certain circumstances RhoB can also be farnesylated by the same GGTase I. The determinants of this differential prenylation and its function still remains unclear. [Armstrong SA, Hannah VC et al., 1995; Adamson P, Marshall CJ et al., 1992] The addition of lovastatin and other

statins to cell cultures results in marked changes in cell morphology, which correlate with the disassembly of actin microfilaments, and that are reversed by the addition of mevalonate. Rho activity is essential for the cytoskeletal changes that occur on the activation of polarised cells and can be inhibited by various prenylation inhibitors including statins, indicating that prenylation is also indispensable for the cytoskeletal effects of Rho. [Garret IR, Chen D et al., 2001]

Rho is also involved in the regulation of calcium sensitivity of smooth muscle, and probably of other cells, that can also be inhibited by statins. [Grönroos E, Andersson T et al., 1996; Alvarez DS and Andriantsitohaina R, 2001] The Rho proteins act as efficient substrates for the *Clostridium botulinum* C3 ADP-ribosyltransferase exoenzyme which ADP-ribosylates and inactivate Rho. This toxin and enzyme is used as an additional tool in the investigation of cytoskeletal assembly and, experimentally, it is applied as an inhibitor to Rho. The effect of this *Clostridium botulinum* exotoxin produces the same cellular morphological changes as those observed with the addition of statins. [Aktories K, 1997] It would also indicate that the pathways affected by statins and *Clostridium botulinum* exotoxin which disrupt the cytoskeleton, are the same. Indeed this supposition is now routinely made when studying these effects.

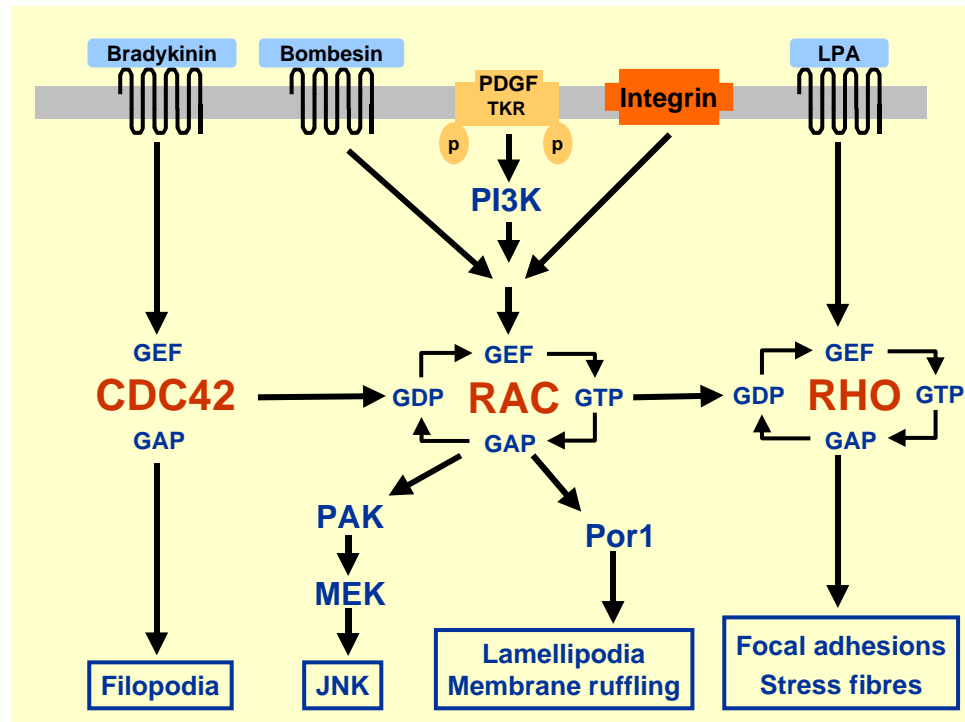
The Rac family of the Rho proteins is involved with actin filament organisation, which leads to the formation of lamellipodia and membrane ruffling induced by growth factors. It is involved at a relatively early stage in the sequence of events during the cytoskeletal organisation that occurs in concert with Rho. This process can be inhibited by the microinjection of inactive Rac mutants and prenylation inhibitors, including statins. [Craig SW and Johnson RP, 1996] Rac also has an influence on the assembly of stress fibres indicating a communication with Rho and Rac. Rac additionally plays an essential role in the NADPH oxidase system of phagocytic leukocytes (neutrophils, macrophages, and eosinophils) which is dependent on prenylation and which can also be prevented by inhibitors of prenylation. [Kreck ML, Freeman JL et al., 1996]

The Rho family of proteins therefore has a profound effect on the cytoskeleton and its dynamics. It can therefore be expected that Rho proteins play an important role in polarised and motile cells such as macrophages. Osteoclasts are another example of such cells, and it is to be anticipated that Rho proteins will play an important role in bone remodeling. Drugs modulating these effects can also be postulated to influence the function of the Rho proteins.





#### 1.4. The involvement of prenylation in bone metabolism.



**Figure 1.8. CDC42, Rac and Rho.**  
 A schematic representation of the signal transduction pathways from the cell surface to the cytoskeleton. The binding of ligands to the serpentine receptors, tyrosine kinase receptors and integrins result in signal cascades for which CDC42, Rac and Rho are pivotal, and which lead to cytoskeletal reorganisation and activation of polarised and motile cells. Note that nuclear transcription factors are also activated.

Motile and polarised cells can be activated by a variety of stimuli; via the ligand binding of the serpentine and tyrosine kinase receptors, and via integrins after contact with components of the extracellular matrix and other cell adhesion molecules (Fig. 1.8). [Denhardt DT, 1996] The activation of cells, and in particular polarised and motile cells such as osteoclasts and monocyte-derived macrophages, by growth factors, cytokines and integrins, requires the transmission of a signal from the cell surface to the cytoskeleton. [Clark EA, King WG et al., 1998] This leads to activation of these cells, changes in the cytoskeletal organisation and results in the formation of filopodia,

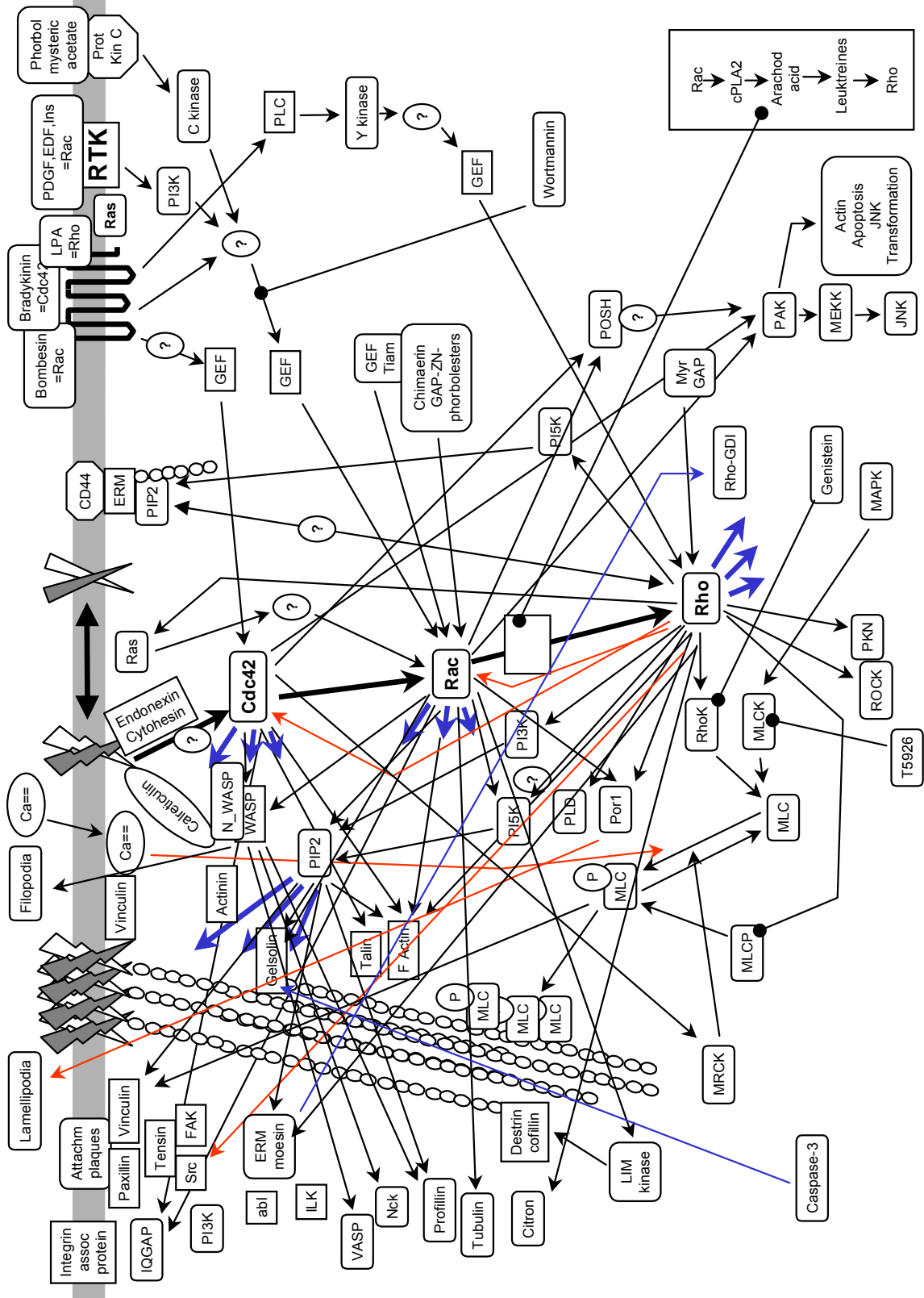
lamellipodia (cell ruffling), and focal adhesion complexes and stress fibres. This in turn results in alterations in cell morphology, and confers mobility to these cells. In parallel with these morphologic changes, certain growth characteristics of the cell are altered – some cells start proliferating or dividing while other cells undergo programmed cell death or apoptosis. The signal transduction pathways from the cell surface to the cytoskeleton can follow different paths and a complex system of cross-talk exists between these different signal transduction pathways (Fig 1.9). [Gauthier RC, Vignat E et al., 1998; Denhardt DT, 1996; Laufs U and Liao JK, 1998; Lim L, Manser E et al., 1996; Reszka AA, Wesolowski G et al., 1998] Consequently, and important to realize that the response to growth factors, cytokines or integrins differs in different cell types. Contact with a particular extracellular matrix protein will cause proliferation in one cell type but may cause apoptosis or death in another cell. [Ghosh PM, Mott GE et al., 1997] This may have important implications for the effects of prenylation inhibitors in bone and mineral metabolism. [Gómez J, Martínez AC et al., 1998]

It is clear that CDC42, Rac and Rho play a central and critical role in cytoskeletal reorganisation. In addition, Rac and Rho, and other elements related to the cytoskeleton, also play a role in transmitting signals to the cell nucleus, leading to transcription and translation (Fig. 1.9). Of note is the important role that PI3 kinase and other phosphatidylinositol kinases play in these pathways, acting as an important link between the receptors and cytoskeletal elements (Fig. 1.9). [Carpenter CL, Toliaas KF et al., 1997; Gómez J, Martínez AC et al., 1998; Martin SS, Rose DW et al., 1996]

Signals which affect the cytoskeleton for the most part involve the Rho family of small GTPases, namely Rho, Rac and CDC42. [Hall A, 1998; Burridge K and Chrzanowska WM, 1996; Tapon N and Hall A, 1997] As indicated, CDC 42 is involved with the formation of filopodia, Rac to lamellipodia and membrane ruffling, and Rho regulates the formation of focal adhesion and stress fibres. [Craig SW and Johnson RP, 1996] After contact with the appropriate ligand, the Rho proteins are activated which,

amongst other processes, involves prenylation and specifically geranylgeranylation, resulting in a translocation of Rho from the cytosol to membranes. The degree of activation and the duration of the signal are further determined by associated modulating proteins which determine the GTPase activity and membrane association. [Ando S, Kaibuchi K et al., 1992; Sasaki T and Takai Y, 1998]





**Figure 1.9. Signaling pathways** between the cell surface and cytoskeletal elements.

Adapted from an extensive literature search.

A predominant overall downstream effect after ligand binding to the serpentine receptors, some of the tyrosine kinase receptors and the integrins is cytoskeletal reorganisation. Prenylation inhibitors including statins block the cholesterol synthetic pathway and reduce the availability of the substrates for prenylation, namely farnesylpyrophosphate and geranylgeranylpyrophosphate. Prenylation inhibitors can block the cytoskeletal effects seen after ligand binding. These blocking effects produced by the prenylation inhibitors can be reversed by the addition of mevalonate and geranylgeranylpyrophosphate but not by farnesylpyrophosphate - this implies involvement of Rho, which is geranylgeranylated, and not Ras, which is farnesylated. Importantly, other downstream products of the cholesterol synthetic pathway, including the addition of LDL-cholesterol, are unable to reverse the effects of the statin prenylation inhibitors. The statins therefore induce their effect on the cytoskeleton via an inhibition of geranylgeranylation.

The Rho proteins are geranylgeranylated and it is logical to assume that they are a target of the statins when the statins affect the cytoskeleton. The inhibitory cytoskeletal effects of the statins can be mimicked by *Clostridium botulinum* C3 transferase exotoxin and *Clostridium difficile* Toxin B, which are inhibitors of Rho, and can also be mimicked by the expression of dominant negative mutations of Rho in the cells. [Laufs U and Liao JK, 1998] *Clostridium botulinum* C3 transferase also prevents the reversal by geranylgeranylpyrophosphate of the cytoskeletal effects produced by the treatment with statins. The cytoskeletal effects of statins can be counteracted by the addition of *Escherichia coli* necrotising exotoxin, an activator of Rho proteins. [Kreck ML, Uhlinger DJ et al., 1994] It is clear therefore, that geranylgeranylation, and as a result Rho, plays a critical role in the downstream events following on signalling which leads to cytoskeletal reorganization. These events can be profoundly affected by prenylation inhibitors such as the statins.

However, there are other downstream effects and effectors of activated Rho that may play an important role in various processes and organs. Nuclear transcription of various proteins may be directly or indirectly affected. [Lim L, Manser E et al., 1996; Denhardt DT, 1996] Furthermore nitric oxide synthase (NOS) is regulated by Rho proteins which act as negative regulators [Laufs U and Liao JK, 1998] , either by increased transcription and/or by prolonged half-life and stability of the NOS mRNA or of the enzyme itself. [Lim L, Manser E et al., 1996]

Osteoclasts are amongst the cells that undergo cytoskeletal organisation and membrane ruffling prior to activation. It has been demonstrated that Cdc42, Rho and Rac proteins are pivotal intermediaries in the signal transduction between the integrins and receptors on the cell surface and actin filament organisation (Fig. 1.8; 1.9). [Craig SW and Johnson RP, 1996] Given the above, there is every reason to believe that inhibition of prenylation should have some effect on osteoclasts and that this effect may be inhibitory.

There is evidence that the ultimate target for bisphosphonates is the osteoclast and that they cause inhibition and apoptosis of osteoclasts, and also inhibit osteoclastogenesis. [Rodan GA, 1998; Luckman SP, Coxon FP et al., 1998a] It has been demonstrated that the nitrogen containing bisphosphonates, including alendronate, inhibit prenylation via the inhibition of farnesyl pyrophosphate synthase. [Luckman SP, Hughes DE et al., 1998; van Beek ER, Pieterman E et al., 1999] This evidence linking osteoclasts, the inhibition of prenylation, and alendronate therefore make it very likely that statins, which have a similar mode of action, would also have an important inhibiting effect on osteoclasts and therefore bone and mineral metabolism. [van Beek ER, Löwik C et al., 1999]

## Chapter 2: Hypothesis, aims and methodology of the studies

At the time of the start of our studies in August 1998, no data were available on the effect of prenylation and statins on bone metabolism and little on the effect of statins on the cytoskeleton. Furthermore, important additional data only became available after the completion of our first animal studies. At the time of the formulation of our hypotheses, the available data seemed to favour a major negative effect of statins on osteoclast function and bone resorption.

### 2.1. Hypotheses

There is evidence to support the notion that osteoporosis and atherosclerosis are linked. On this basis lipid lowering therapy could therefore be expected to also impinge on processes in bone. There is also a large amount of data available that indicates that prenylation plays an important role in osteoclast function and bone metabolism. Alendronate inhibits osteoclast function and alendronate has also been shown to inhibit prenylation. It would therefore be reasonable to assume that the inhibition of prenylation by statins might have a similar effect on bone resorption and/or formation and ultimately bone health. Data from lipid metabolism and from the pharmacokinetics of various statins seemed to indicate that the effects of different statins are not the same. There was also some evidence to suggest that the pleiotropic effects of the different statins are not the same.

It was therefore hypothesized that statins would have some effect on bone metabolism and that this should be investigated. It was also imperative to formulate sound hypotheses based on information existing at the time, and to design studies to prove or disprove these hypotheses.

The following generalised hypotheses were therefore postulated:-

- Statins will have an influence on bone and mineral metabolism
- Similar to alendronate, statins will inhibit osteoclast function
- Statins will increase bone mineral density
- The effect of statins on bone will be the greatest in experimental models of high bone turnover e.g. oestrogen-deprived animals.
- The effect on bone may differ between different statins

## **2.2. Aims of the studies.**

The aims of the studies were the following:-

- To investigate the effect of simvastatin on bone mineral density (BMD) in intact and ovariectomised rats
- To investigate the effect of simvastatin on quantitative bone histomorphometry (QBH) including parameters of bone resorption and formation, in intact and ovariectomised rats
- To investigate the effect of different dosages of simvastatin on BMD and QBH in intact rats
- To investigate the effect of other statins (pravastatin, atorvastatin) on BMD in intact rats.



### **2.3. Methodology for the studies in rats**

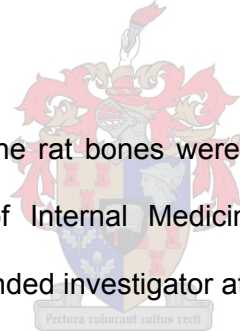
The studies on the rats utilised a uniform methodology to be described in this chapter. Slight variations in procedure between experiments are described where relevant.

#### **2.3.1. Sites of the studies**

The rats were in the Animal Research Unit of the Faculty of Health Sciences of the University of Stellenbosch located at Tygerberg in the Western Cape Province.

The surgical procedures on the rats were performed in the Animal Research Unit of the Department of Anatomy of the Faculty of Health Sciences, University of Stellenbosch, Tygerberg.

BMD measurements on the rat bones were performed in the Endocrinology and Metabolism Unit, Department of Internal Medicine, Ward A10, Tygerberg Hospital, Tygerberg and confirmed by a blinded investigator at the University of Pretoria.



QBH was performed in the Bone Histology Laboratory of the above Endocrinology and Metabolism Unit, Department of Internal Medicine, Ward A10, Tygerberg Hospital, Tygerberg.

The biochemical measurements of the rat follicle stimulating hormone (rFSH) were performed in the Department of Chemical Pathology, Faculty of Health Sciences, Tygerberg Hospital, Tygerberg.

The measurements of serum oestradiol were performed in the Department of Chemical Pathology, University of Pretoria, Pretoria.

### **2.3.2.. Ethical approvals, registrations and time schedules**

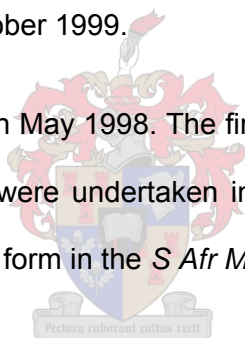
The Research C Subcommittee of the Ethics Committee, and the Animal Research Committee, Faculty of Health Sciences, University of Stellenbosch, approved the treatment and study protocols:-

- Study and approval number: 98/131
- Approval date: 30 October 1998.

The studies were registered for a Doctoral thesis for Dr Frans J Maritz with the Registrar of the University of Stellenbosch:-

- Approval date: 22 October 1999.

The studies were started in May 1998. The first results of Study 3.1 were available in August 1998. Further studies were undertaken in April 1999 and the first preliminary results were published in abstract form in the *S Afr Med J* 1999; 879: 478.



### **2.3.3. Materials**

Simvastatin (Zocor; Merck, Sharpe & Dohme), atorvastatin (Lipitor; Parke-Davis) and pravastatin (Prava; Bristol-Myers Squib) were obtained commercially.

The serum rat FSH (rFSH) assay system (Biotrak; rFSH [<sup>125</sup>I], code RPA550, Amersham Life Science Ltd, Buckinghamshire) was obtained from AEC Amersham, South Africa.

Diagnostic Product Corporation, South Africa supplied the oestradiol kit (Estradiol double antibody).

The rat feeds (Rat and Mouse Breeder Feed; Animal Specialties (PTY) Ltd; Phosphorus (min) 8g/Kg, calcium (max) 18g/Kg.) were provided by the Animal Research

Unit, Faculty of Health Sciences, University of Stellenbosch. Oxytetracycline hydrochloride (Terramycin 100; Pfizer Animal Health) was obtained commercially.

#### **2.3.4. The general rat model**

The female Sprague-Dawley rats were all acquired from the Animal Research Unit, Faculty of Health Sciences, University of Stellenbosch. For all the studies, three-month-old female rats weighing approximately 250gm were obtained from similarly raised and weaned litters, and housed, 5 rats per cage, in a light (14h) and temperature (23-25<sup>0</sup>C) controlled environment in a pathogen free room. The rats were allowed free access to water, were pair-fed and weighed bi-weekly and feeds adjusted to keep the weights constant.

Rats were randomly allocated to groups of ten rats each. Rats receiving active medication were compared to a control, placebo-treated group. The rats on active medication received their respective statin, dissolved in vegetable oil as vehicle and mixed in their feeds, while the control groups received only the vehicle vegetable oil as placebo. In all other respects the actively treated rats and the rats in the control groups were treated and managed identically.

The duration of treatment before sacrifice was 8 weeks in the ovariectomy/sham model and 12 weeks in all the other rat studies.

In all the groups of rats, 13 days and 3 days before sacrifice, all animals received oxytetracycline hydrochloride (25mg/Kg, intramuscularly). At the end of the study periods the rats were sacrificed using thiopental, and the tibias and femurs were harvested for quantitative bone histomorphometry and bone mineral density measurements respectively.

### **2.3.5. Bone mineral density**

For the BMD measurements the femurs were preserved in 70% alcohol. BMD of the right femur of each rat was measured employing dual energy x-ray absorptiometry (Hologic QDR 1000), utilising the software and methodology provided by Hologic Inc.

The BMD measurements performed on the femurs of the ovariectomy model were repeated on a separate Hologic QDR1000 densitometer at a different center (University of Pretoria), using the same methodology and software, and the results were then compared.

### **2.3.6. Quantitative bone histomorphometry**

For the QBH estimations, one tibia from each rat was removed, fixed in a modified Millonig's solution (3.7% formaldehyde, 93mm NaH<sub>2</sub>PO<sub>4</sub>, 105mm NaOH and 14.6mm sucrose) for 24 hours only, embedded in methylmethacrylate, sectioned at 5µm and stained by the Goldner technique. [Jones R and McClung A, 1990] QBH analyses were performed, using a Merz-Schenk integrating eyepiece, [Merz WA and Schenk RK, 1970] by a single, experienced technician blinded to the treatment group of the rats.

Trabecular bone only was analysed, by not including sections within 2 fields (x 250 magnification) from either the growth plate or the cortices. Particular care was taken to analyze this same, standardized site in every animal. At least 120 fields per animal were counted. Time-spaced tetracycline labeling was assessed on unstained, 50µm thick sections. Histomorphometry terminology and calculations used are those described in the Report of the American Society for Bone and Mineral Research Committee on Histomorphometry Nomenclature. [Parfitt AM, Drezner MK et al., 1987]

### **2.3.7. Data.**

For each study the raw data for that particular study will be presented as an appendix. Data pertinent to the discussion of any particular study will be presented as a table in the relevant chapter. For illustrative purposes data will, where possible, be presented in graphic format..

### **2.3.8. Statistics**

For the statistical analysis, and for all the studies, the BMD measurements and QBH parameters were compared to their respective controls. Further between-group analyses were done where appropriate.

Traditionally the differences between groups are examined by means of a Student's t-test. A Student's t-test assumes that the data has a normal distribution and was designed specifically to examine small sample sizes of biological data.

Much of the data on bone mineral density and quantitative bone histomorphometry in our studies followed a normal distribution and initially differences between groups were examined using the Student's t-test. However, with sample sizes of 10 or less, even when the data appears to have a normal distribution, a normal distribution cannot automatically be inferred and a non-parametric method of examining the difference between samples must be used. The use of the Mann-Whitney U-test is advised under these circumstances. [Dineen LC and Blakesley LC, 1973; Siegel S, 1956] The Mann-Whitney U test assumes that the variable under consideration was measured on at least an ordinal (rank order) scale. The interpretation of the test is essentially identical to the interpretation of the result of a Student's t-test for independent samples, except that the computation of the U test is based on rank sums rather than means of the samples. The U test is the most powerful (or sensitive) non-parametric alternative to the t-test for independent samples; in fact, in some instances it may offer even greater power to reject the null hypothesis than

the t-test. Therefore in these studies, a Mann-Whitney U-test for independent samples was used to examine the differences between the groups.

Where multiple parameters are analysed and are compared with each other it is not correct to analyse each parameter individually and in isolation. Consideration should be given to the influence of other parameters on the findings of any individual parameter. For this reason, an overall comparison of all the groups must be made and the results must be analysed by ANOVA. Accordingly, between groups analyses should also be performed using some form of post hoc analysis within ANOVA. However, it may also be argued that ANOVA is not appropriate for the analysis of the small biological samples as presented here.

In view of these considerations, additional statistical analyses of the BMD and QBH data were made utilising ANOVA. Differences between groups and comparisons with controls were analysed with a post hoc analysis with Fisher's protected least significance difference (PLSD) test.

Since there were no differences between results obtained with ANOVA plus Fisher's PLSD Mann-Whitney U-test, the statistical figures quoted in the text will be from results obtained from the analyses using the Mann-Whitney U-test. The results of the ANOVA and other statistical analyses will be available in the Appendices that contain the descriptive statistics for the different groups used in the different studies. These appendices are numbered and labelled with numbers that correspond with the numbers of the individual studies.

A correlation between the different doses of simvastatin and the QBH parameters of bone formation and resorption was examined by Pearson's test. All statistical analyses were performed by computer utilising Statistica software, Kernel release 5.5 A.

The results of statistical analyses performed in each group will be presented in the appendix section. Other statistical data will be quoted in the text or in tables where applicable.

### **2.3.9. Financial support.**

The research was funded from the following sources: -

- A Research Grant from the Harry Crossley Trust, University of Stellenbosch. Approval for this grant was given on 2 December 1998 and a further grant was given in 1999. The monies and funds were managed by the Faculty of Health Sciences of the University of Stellenbosch.
- Personal funds of Dr Frans Maritz

No financial support or otherwise was received from the pharmaceutical industry for the completion of these studies. No financial support of kind was received or accepted from the Pharmaceutical Industry for the presentation of this data at National Congresses.

### **Chapter 3: Studies on the effect of statins on bone and mineral metabolism.**

The following studies were performed in rats utilising sham-operated and ovariectomised rats, and also different doses of statins, as well as different statins, in intact rats:-

- The effect of simvastatin 20mg/Kg/day administered for 8 weeks on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.
- The effect of simvastatin 20mg/Kg/day administered for 12 weeks on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.
- The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.
- The effect of atorvastatin 2.5mg/Kg/day and pravastatin 10mg/Kg/day administered for 12 weeks on bone mineral density in intact female Sprague-Dawley rats.

The studies were performed to answer specific questions based on a pre-existing formulated hypothesis. The hypotheses were based on sound data available at the time of the planning of these studies. The studies are presented separately in sub-chapters of this chapter. The data for each study are presented in the form of summary tables and in figures. The tables and figures are grouped into the separate sub-chapters of the relevant



studies. The tables and figures are labeled according to the relevant sub-chapter number for ease of reference.

For sake of brevity and to avoid unnecessary repetition, in the introductory background section of each study on which the hypotheses were based, reference will be made to background information presented in Chapter 1.

The complete data with the relevant descriptive statistics and the statistical analyses are presented and available in the Appendices section. The appendices are numbered according to the study concerned.



### **3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.**

#### **3.1.1. Background**

At the start of these studies little was known regarding the effect of prenylation or statins on bone and mineral metabolism.

As described in Chapter 1, there is sufficient information to suggest that more than a casual link exists between osteoporosis and atherosclerosis. [Parhami F, 2000] This suggests that the treatment of dyslipidaemia might have an effect on the associated osteoporosis, or at least have some effect on bone metabolism.

As early as 1995 there was an indication that lipid lowering agents might have an effect on maintaining bone mass. [Wang GJ, Chung KC, and Shen WJ, 1995] Three groups of rabbits were treated with glucocorticoids, two of which also received lovastatin or bezafibrate. After 13 weeks the histologic trabecular bone area was higher in the groups that had lipid-lowering agents compared to the group that receive steroid only. It was therefore concluded that lipid-lowering agents could prevent steroid-induced osteoporosis and that this might be an additional use of these agents. The use of lovastatin in these studies was the first indication that statins might have an effect on bone metabolism. Further work by these researchers supported their earlier findings. They showed that lovastatin could prevent the effect of steroids on adipogenesis in cultured cells; lovastatin inhibited steroid induced fat-specific gene expression in cultured marrow cells and counteracted the inhibitory effects of steroids on osteoblastic gene expression. [Cui Q, Wang GJ et al., 1997] They also showed that lovastatin was able to prevent steroid-induced osteonecrosis in chickens. The authors therefore concluded that lovastatin might have a role in the prevention of osteonecrosis.

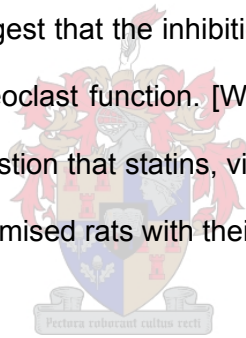
The critical and indispensable link between ligand binding to integrins and certain cell receptors, and cytoskeletal activation with the involvement of Rac and Rho on the one hand and the activation of polarised and motile cells such as osteoclasts on the other hand, has been established and alluded to. [Craig SW and Johnson RP, 1996; Giancotti FG, 1997; Zigmund SH, 1996; Hall A, 1998; Symons M, 1996] There is ample evidence that prenylation inhibitors including statins can inhibit the function of Rac and Rho. This evidence is, on the one hand, direct, where the inhibition of Rac or Rho by a statin has been primarily demonstrated. [Hughes AD, 1996; Lebowitz PF, Casey PJ et al., 1997] On the other hand the evidence is indirect, where statins have been used in numerous experiments as a control to inhibit the effect of Rho function and cytoskeletal organisation. [Kranenburg O, Poland M et al., 1997] This evidence alone suggests that the use of statins will have some effect on cells involved in bone turnover, such as osteoclasts.

The evidence linking protein prenylation and osteoclast function only became apparent in an indirect fashion. It was demonstrated that bisphosphonates including alendronate inhibited osteoclast function by suppressing osteoclastogenesis, inhibiting osteoclast function and causing apoptosis of osteoclasts. [Rogers MJ, Chilton KM et al., 1996; Sato M, Grasser W et al., 1991; van Beek ER, Löwik CW, and Papapoulos SE, 1997] Subsequently it was demonstrated that alendronate inhibits the mevalonate pathway and that it inhibits prenylation. This inhibition of prenylation was accordingly demonstrated to be the mode of action of alendronate. [Luckman SP, Hughes DE et al., 1998; Luckman SP, Coxon FP et al., 1998a; Luckman SP, Coxon FP et al., 1998b] Indeed it was later shown by the Dutch group that alendronate inhibits isopentenyl pyrophosphate isomerase/farnesol pyrophosphate synthase activity. [van Beek ER, Pieterman E et al., 1999; van Beek ER, Löwik C et al., 1999] In some of these initial experiments mevastatin was used as a control and produced an effect similar to that seen with alendronate and could inhibit osteoclast function. [Luckman SP, Hughes DE et al., 1998]

Further suggestive evidence came from the effect of statins on certain cell lines. Statins were able to inhibit certain aspects of macrophage function in blood vessels. [Bellosta S, Bernini F et al., 1998] It was shown that lovastatin was able to induce apoptosis in mesangial cells. [Ghosh PM, Mott GE et al., 1997] Macrophages, mesangial cells and osteoclasts are all motile cells that are derived from the same lineage. It therefore seemed reasonable to assume that the effects of statins on macrophages and mesangial cells would extend also to osteoclasts.

The above suggested that statins will have an effect on bone turnover and in particular on osteoclast function. This prompted us to pursue this line of enquiry further.

Oestrogen deprived animals are known to have a high bone turnover state. The existing evidence seemed to suggest that the inhibition of prenylation via alendronate and also via statins would inhibit osteoclast function. [Woo JT, Kasai S et al., 2000] These factors led credence to the suggestion that statins, via their inhibition of prenylation, would have a greater effect on ovariectomised rats with their high-turnover state than their sham-operated counterparts.



### **3.1.2. Hypothesis**

Based on the above information, the following hypotheses were formulated: -

- Simvastatin 20mg/Kg/day administered for 8 weeks will affect bone and mineral metabolism
- Simvastatin 20mg/Kg/day administered for 8 weeks will decrease osteoclast function and consequently reduce bone resorption
- Simvastatin 20mg/Kg/day administered for 8 weeks will increase BMD

Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

- The effect of simvastatin 20mg/Kg/day, administered for 8 weeks, on QBH parameters of bone resorption and formation, as well as BMD, will be greater in ovariectomised rats than in their sham-operated controls

### **3.1.3. Aims of the study**

The study was aimed to investigate the following:-

- To investigate the effect of simvastatin 20mg/Kg/day for 8 weeks on BMD and on QBH parameters of bone resorption and formation in sham-operated and ovariectomised female Sprague-Dawley rats.
- To compare the effects of simvastatin 20mg/Kg/day on BMD and parameters of QBH between sham-operated intact rats and ovariectomised rats.

### **3.1.4. Methodology**

The general rat model, with the associated handling of the rats, feeding, weighing, method of drug and placebo administration, time-spaced tetracycline marking, sacrifice and harvesting of bones was utilised as described in chapter 2.3.4.

Forty rats were randomly allocated to four groups of ten rats each. Two weeks prior to the administration of the study drugs, an ovariectomy was performed under ether anesthesia on two groups. One of these groups received simvastatin 20mg/Kg/day dissolved in vegetable oil as vehicle (OVX-S), while an equivalent amount of vehicle was administered to the other group as placebo (OVX). A sham operation was performed under ether anesthesia on the remaining two groups of which one group received simvastatin 20mg/Kg/day (Sh-S) and the other placebo as above (Sh). The treatment was continued for 8 weeks in all the groups. The dosages of simvastatin were based on earlier safety and efficacy studies in rats [Gerson RJ, MacDonald JS et al., 1989] and were

similar to those used to assess the effect of statins on bone. [Mundy G, Gutierrez G et al., 1998; Mundy G, Garrett R et al., 1999]

The methodology for quantitative bone histomorphometry and bone mineral density measurements as described in chapter 2.3.5 was utilised. In addition the bone mineral density measurements were repeated at another centre located at the University of Pretoria.

At the time of sacrifice, blood was taken for measurement of rFSH and oestradiol to assess the efficacy of the ovariectomies. rFSH was determined using a competitive [<sup>125</sup>I] assay system with magnetic separation as described by Amersham Life Sciences Ltd. for the assay system (Biotrak; rFSH [<sup>125</sup>I]). Oestradiol was measured by a double antibody method on an Immuno1 analyser.

The results of the BMD and the QBH in the sham-operated group (Sh) were compared to the ovariectomised group (OVX). The results of the actively treated groups (Sh-S, OVX-S) were compared to the placebo treated controls (Sh, OVX) respectively. The delta values for the BMD and the different parameters of bone formation and resorption in the sham-operated group were compared to the BMD and corresponding parameters in the ovariectomised rats.

### 3.1.5. Results

The descriptive statistics of the hard data and the results of the statistical analyses are presented in Appendix A 3.1.

The BMD, employing DEXA, was decreased in the ovariectomised rats (OVX) when compared to the sham-operated animals (Sh) (Fig. 3.1.1; Table 3.1.1). Similarly, bone volume, when employing QBH was decreased in the ovariectomised rats (OVX) when compared to the sham-operated group (Sh) ( $p = 0.00037$ ) (Table 3.1.1). In addition, the QBH parameters of bone resorption were increased in the ovariectomised rats (OVX) (Figs. 3.1.2; 3.1.4; Table 3.1.1) and there was an increase in QBH parameters of bone formation (Figs. 3.1.3; 3.1.4; Table 3.1.1), including the bone formation rate (Table 3.1.1), in the ovariectomised animals when compared to their sham-operated controls (Sh). These expected effects of ovariectomy on BMD and QBH tend to validate the rat model used in this study.

In the sham-operated rats that received simvastatin (Sh-S), the BMD showed a tendency to be lower when compared to their untreated controls (Sh) but this never reached statistical significance (Table 3.1.1; Fig 3.1.5). The addition of simvastatin to the ovariectomised animals (OVX-S) produced no change in the BMD when compared to their untreated controls (OVX) (Table 3.1.1; Fig 3.1.5). However, simvastatin produced a significantly greater effect and decline (delta) in the BMD of the sham-operated group (Sh-St) than in the ovariectomised group ( $p = 0.003$ ) (Fig. 3.1.6). It is also evident that treatment with simvastatin 20mg/Kg/day was unable to prevent the decline in BMD seen in the ovariectomised group (OVX-S) (Fig. 3.1.5).

The static parameters of bone formation (osteoid volumes, surfaces, osteoblasts) were significantly increased in the sham-operated animals which received simvastatin (Sh-S) supporting previous reports that statins increase bone formation (Fig. 3.1.7.).

[Mundy G, Gutierrez G et al., 1998] This was, however, not supported by dynamic, tetracycline-based data and the calculated bone formation rate was similar in the sham-operated animals which did and did not receive simvastatin (Sh-S and Sh) (Fig. 3.1.7.). Reasons for this discrepancy are unclear. Hyperosteoidosis could not be ascribed to a mineralisation defect and the mineralization lag time was unaffected statin administration (Table 3.1.1.). Surprisingly, parameters of bone resorption (eroded surfaces, osteoclasts) were also significantly increased in the statin treated sham-operated rats (Sh-S) (Fig. 3.1.8.). [Mundy G, Garrett R et al., 1999]

In the ovariectomised rats that received simvastatin (OVX-S), the effects of simvastatin on QBH parameters when compared to their untreated controls (OVX) differed from those seen in the sham-operated rats (Sh-S) (Table 3.1.1). The effect of simvastatin 20mg/Kg/day on the formative parameters in the ovariectomised rats (OVX-S) was smaller than that seen in the sham-operated rats (Sh-S), and were not significant (Table 3.1.1). Simvastatin had no effect on the parameters of bone resorption in the ovariectomised rats (Sh-S) (Table 3.1.1). There were no associated changes in bone volume and the changes in bone formation rate were negligible (Table 3.1.1). The differences in QBH parameters of bone turnover in the simvastatin-treated animals (Sh-S and OVX-S) when compared to their respective untreated controls (Sh and OVX), the delta value, differed significantly between the Sh-S and OVX-S groups (Fig. 3.1.9; 3.1.10)

The descriptive statistics and statistical analyses of the data on bone mineral density the descriptive statistics of the data on bone histomorphometry, and the statistical analyses on the bone histomorphometry data are presented in Appendices section (Append. A 3.1.).

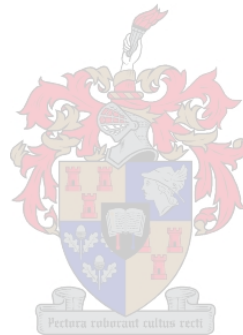
The results of the bone mineral density measurements performed at Pretoria University showed no significant differences from those performed at the University of Stellenbosch.



Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

The oestradiol levels were significantly decreased and the rFSH levels were significantly increased in the ovariectomised (OVX, OVX-S) animals when compared to their sham-operated controls (Sh, SH-S) (Table 3.1.2) indicating that the ovariectomy had been successful.

The rats had a variable weight over the duration of the study and there was a mean weight gain of 22.2 g over the duration of the study (Fig. 3.1.11; Append. A 3.1). However, the weight gain in all the groups was similar and moved in parallel and the weight gain did not differ statistically between groups (Append. A 3.1).



Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### 3.1.6. Tables

**Table 3.1.1. Bone Mineral Density and Histomorphometry: Ovariectomy and Sham-operated Groups.**

	Animal group			
	Sh	Sh-S	OVX	OVX-S
<b>Bone Mineral Density</b>	0.104(0.001)	0.099(0.002)	0.094(0.001)	0.094(0.002)
<b>Histomorphometric parameter</b>				
Bone volume (BV/TV) (%)	18.02 (1.05)	17.29 (1.29)	10.54 (0.88)	9.53 (1.17)
Osteoid volume (OV/BV) (%)	0.8 (0.26)	1.55 (0.3)	2.32 (0.41)	2.63 (0.57)
Osteoid volume (OV/TV) (%)	0.13 (0.03)	0.26 (0.05)	0.23 (0.03)	0.22 (0.04)
Osteoid surface (OS/BS) (%)	4.41 (1.12)	9.53 (1.38)	11.54 (1.7)	13.59 (1.91)
Osteoblast surface (Ob.S/BS) (%)	0.49 (0.11)	1.12 (0.16)	0.77 (0.19)	1.38 (0.39)
Osteoid thickness (O.Th) (mcm)	7.66 (0.98)	7.05 (0.61)	9.61 (1.18)	8.15 (0.83)
Eroded surface (ES/BS) (%)	6.05 (0.94)	8.11 (0.68)	7.94 (1.23)	8.15 (0.83)
Osteoclast surface (Oc.S/BS) (%)	0.74 (0.12)	1.21 (0.14)	1.67 (0.31)	1.69 (0.25)
Osteoclast number (N.Oc/TA) (/mm <sup>2</sup> )	0.06 (0.01)	0.11 (0.01)	0.16 (0.03)	0.16 (0.02)
Mineralizing surface (MS/BS) (%)	5.1 (0.78)	5.18 (0.72)	9.28 (0.83)	8.83 (1.01)
Mineralisation lag time (mlt) (days)	0.59 (0.12)	0.54 (0.08)	0.32 (0.04)	0.27 (0.04)
Bone formation rate (BFR/BS) (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	15.45 (2.52)	15.65 (2.22)	33.37 (4.47)	31.93 (3.5)

	Animal groups compared					
	Sh vs. OVX		Sh vs. Sh-S		OVX vs. OVX-S	
	* %	p	** %	p	*** %	p
<b>Bone Mineral Density</b>	-9.5	<b>0.0065</b>	-4.1	0.1986	-0.06	1.0295
<b>Histomorphometric parameter</b>						
Bone volume (BV/TV) (%)	-41	0.0003	-4	0.8421	-10	0.1128
Osteoid volume (OV/BV) (%)	190	0.0019	94	0.0279	13	0.9048
Osteoid volume (OV/TV) (%)	75	0.0244	103	0.0220	-2	0.9048
Osteoid surface (OS/BS) (%)	162	0.0012	116	0.0133	18	0.3562
Osteoblast surface (Ob.S/BS) (%)	57	0.3401	127	0.0133	78	0.4967
Osteoid thickness (O.Th) (mcm)	25	0.2973	-8	0.6038	-15	0.6607
Eroded surface (ES/BS) (%)	31	0.2224	34	0.0435	3	1.0318
Osteoclast surface (Oc.S/BS) (%)	126	0.0315	64	0.0435	2	0.9682
Osteoclast number (N.Oc/TA) (/mm <sup>2</sup> )	178	0.0078	92	0.0030	0	0.8421
Mineralizing surface (MS/BS) (%)	82	0.0040	2	0.9682	-5	0.6607
Mineralisation lag time (mlt) (days)	-46	0.0244	-9	0.9048	-15	0.4002
Bone formation rate (BFR/BS) (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	116	0.0040	1	0.7802	-4	0.9048

Data expressed as mean (SEM); Sh = Sham; Sh-S = Sham + simvastatin 20mg/Kg/dy; OVX = ovariectomy; OVX-S = ovariectomy + simvastatin 20mg/Kg/dy; \* = % change of OVX from Sh; \*\* = % change of Sh-S from Sh; \*\*\* = % change of OVX-S from OVX. P value = post hoc ANOVA, Fisher's test.

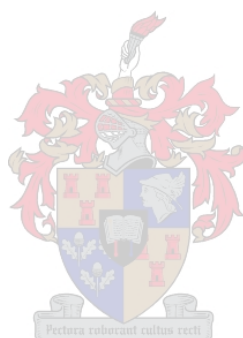
Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

**Table 3.1.2.** Serum rFSH and oestradiol.

	rFSH (ng/ml)	Oestradiol (pmol/L)
Sh	0.6 (0.07)	63 (17.22)
Sh-S	0.51 (0.05)	52.3 (14.41)
OVX	6.5 (0.44) †	15.4 (2.36) †
OVX-S	5.46 (0.25) †	11.81 (1.68) †

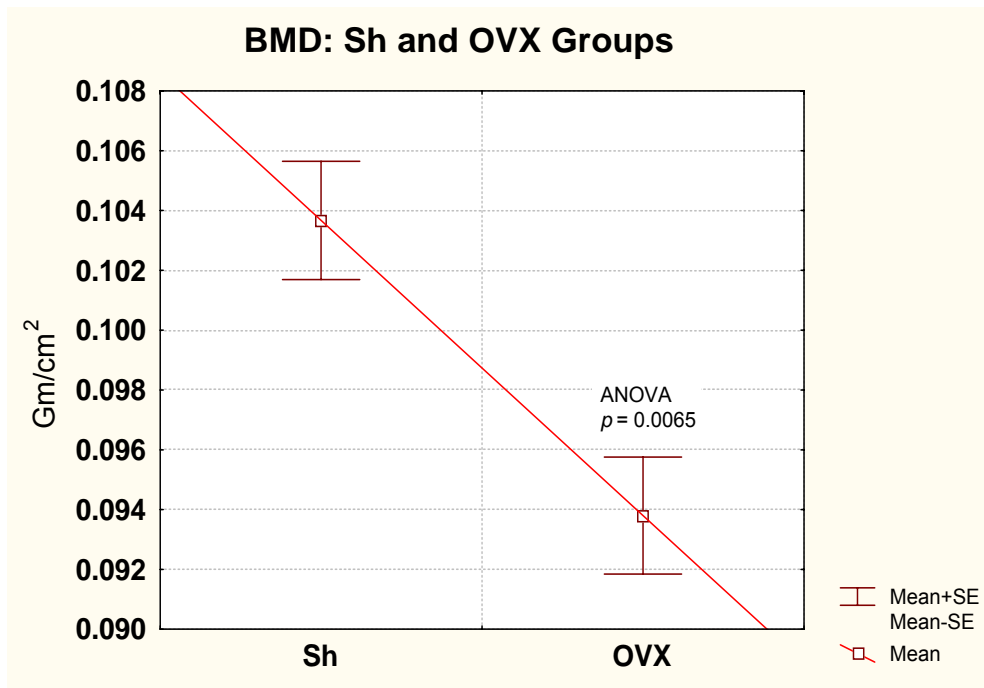
\* Data expressed as mean (SE)

†  $p = <0.001$  compared to Sham group.

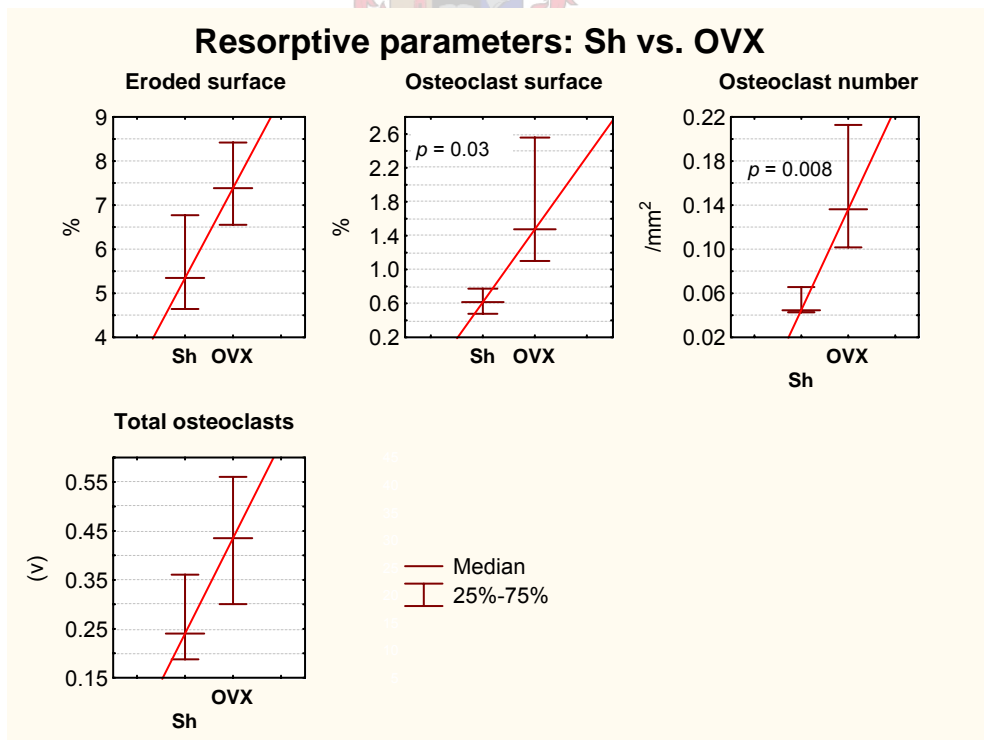


Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### 3.1.7. Figures

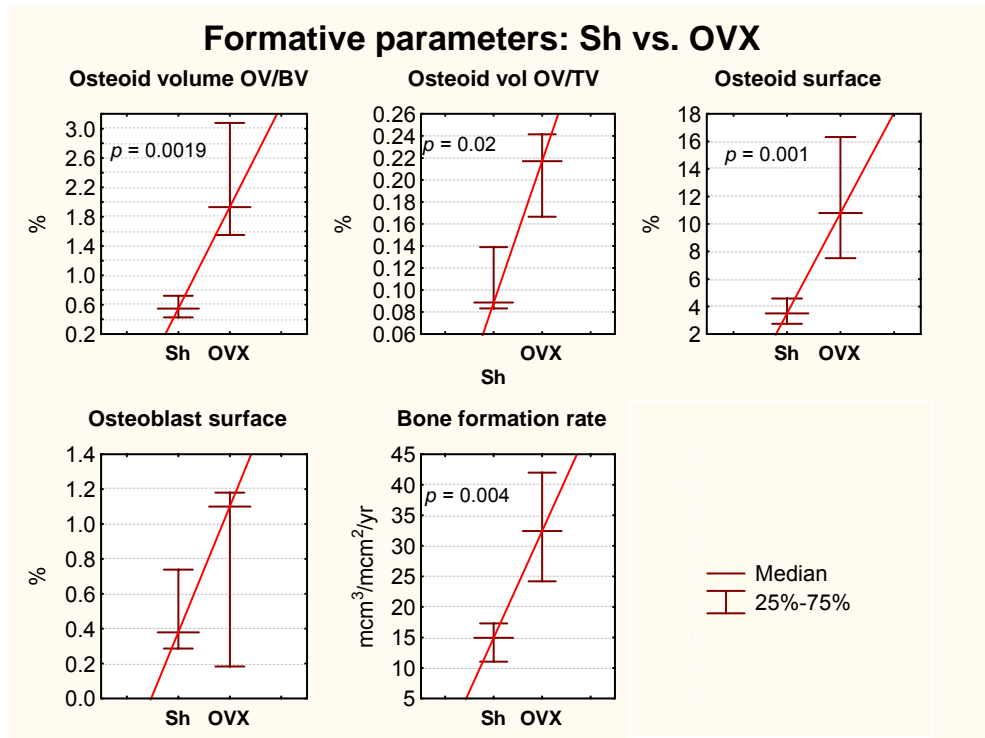


**Figure 3.1.1.** BMD of untreated ovariectomised (OVX) and sham-operated rats (Sh).

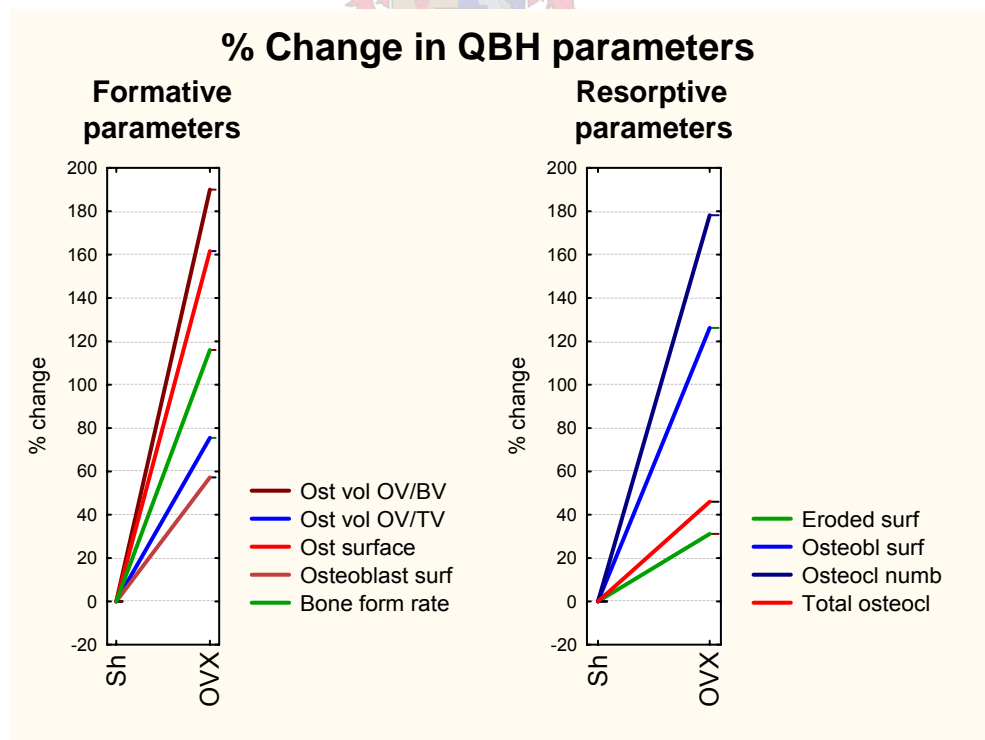


**Figure 3.1.2.** Quantitative bone histomorphometric parameters of bone resorption in untreated ovariectomised rats (OVX) vs. sham-operated controls (Sh).

Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

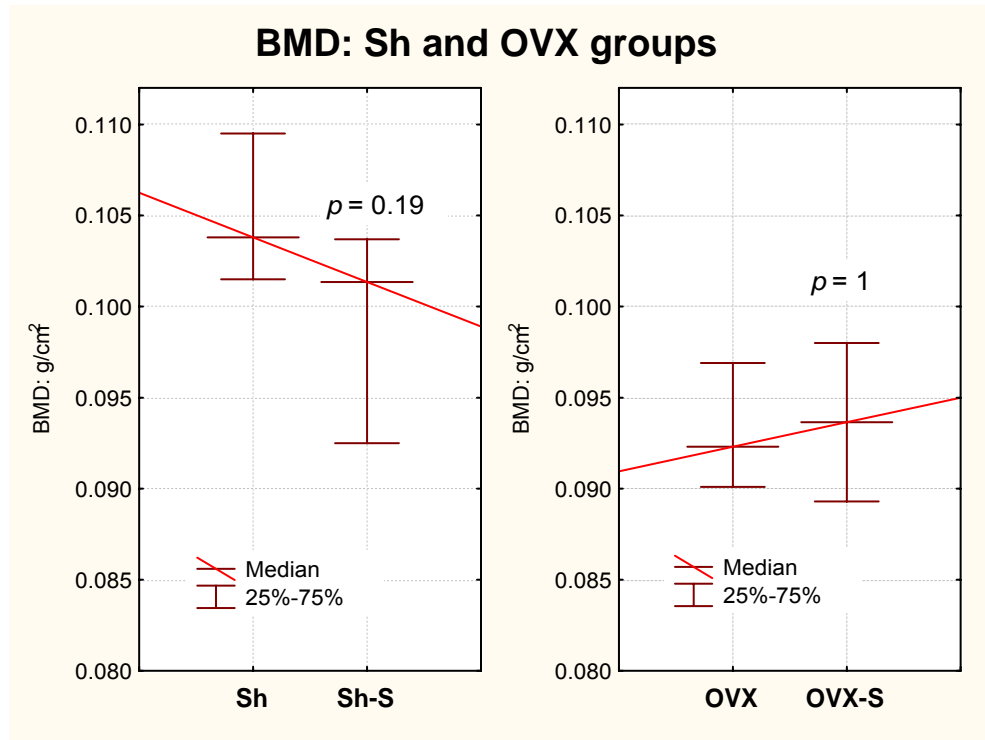


**Figure 3.1.3.** Quantitative bone histomorphometric parameters of bone formation in untreated ovariectomised rats (OVX) vs. sham-operated controls (Sh).

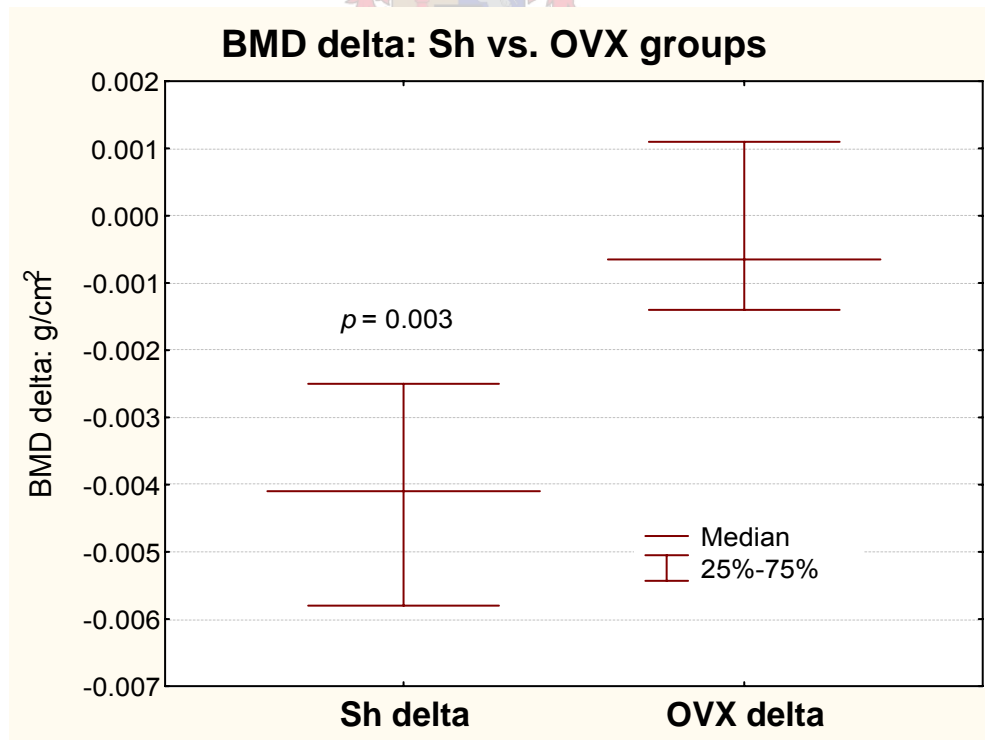


**Figure 3.1.4.** Changes in quantitative histomorphometric parameters of bone formation and resorption in the untreated ovariectomised rats (OVX) expressed as a percent change from their untreated sham-operated controls (Sh).

Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

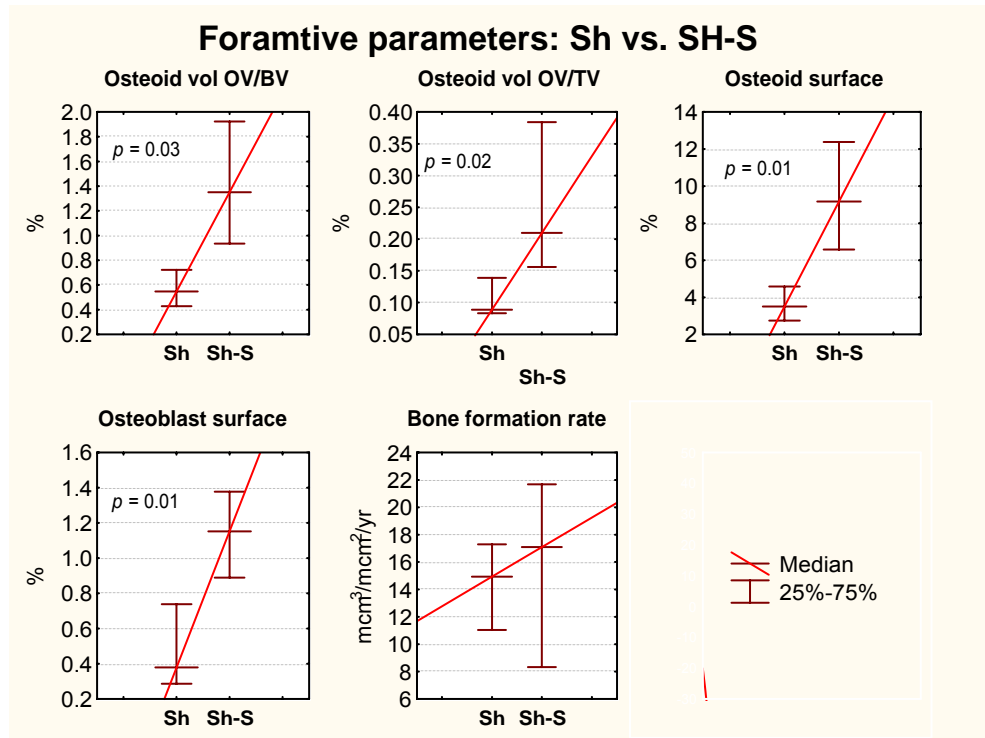


**Figure 3.1.5.** BMD in the sham-operated and ovariectomised rats (Sh and OVX) and in those receiving simvastatin 20mg/Kg/day (Sh-S and OVX-S).

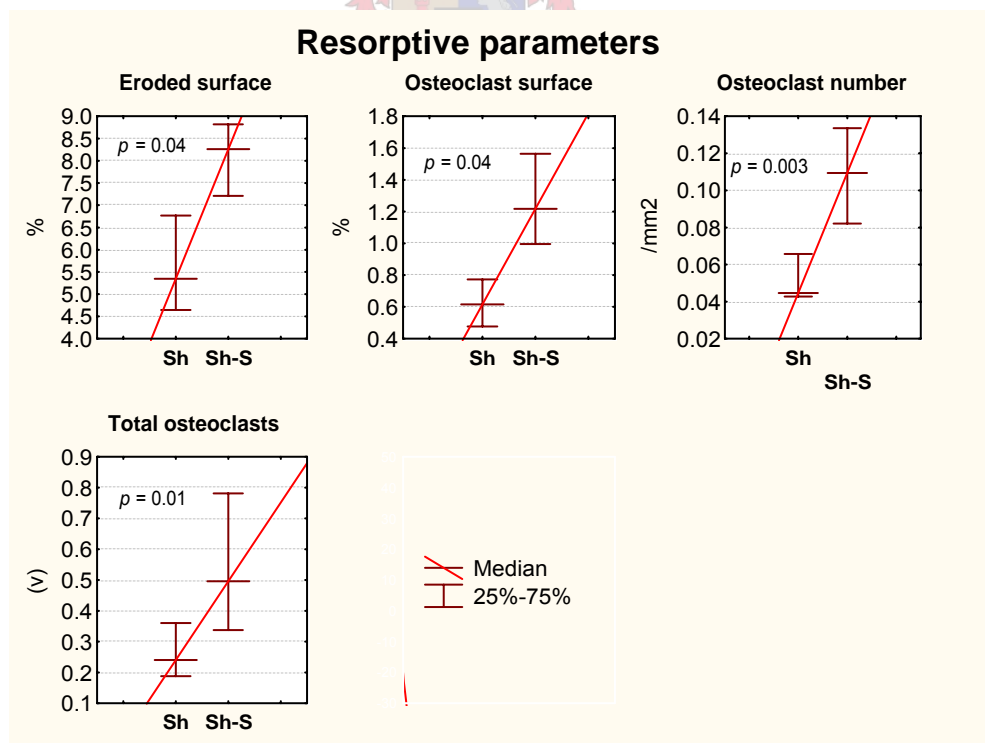


**Figure 3.1.6.** The delta BMD: the change in BMD induced by simvastatin in the Sh and OVX groups.

Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

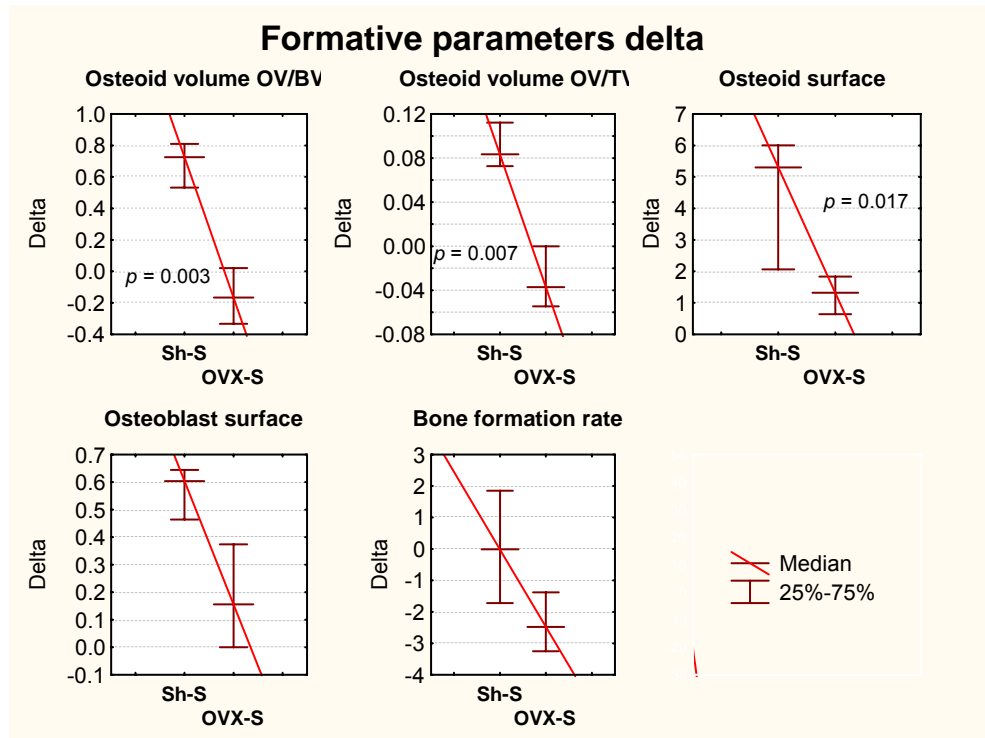


**Figure 3.1.7.** Quantitative histomorphometric parameters of bone formation in the untreated sham-operated rats (Sh) vs. those receiving simvastatin 20mg/Kg/day (Sh-S)

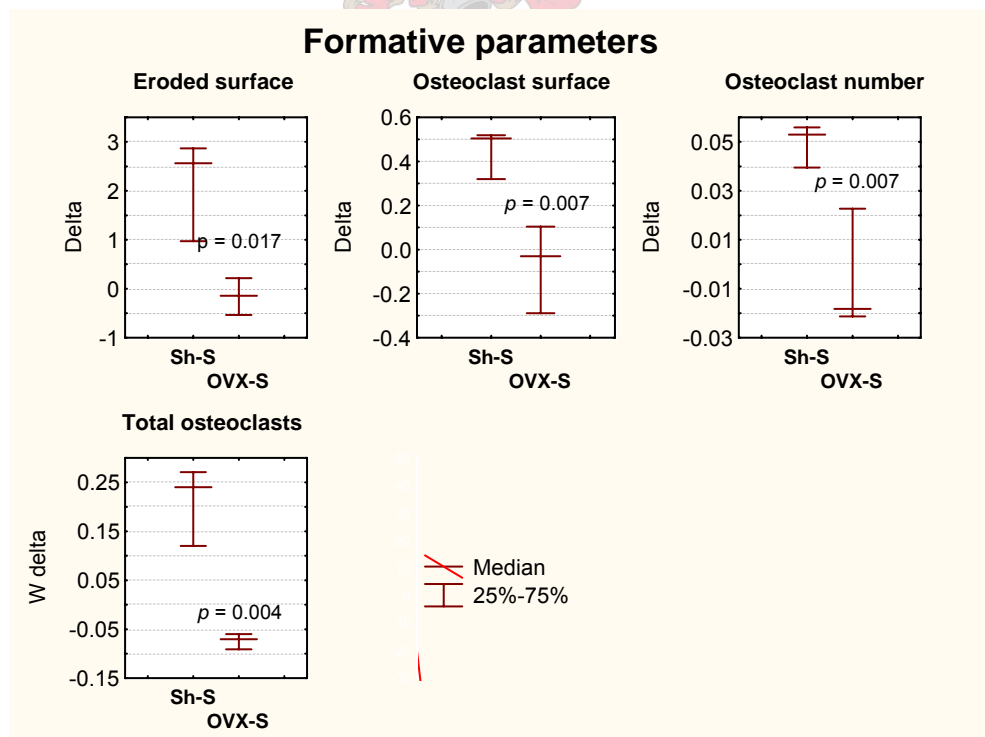


**Figure 3.1.8.** Quantitative histomorphometric parameters of bone resorption in the untreated sham-operated rats (Sh) vs. those receiving simvastatin 20mg/Kg/day (Sh-S).

Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.



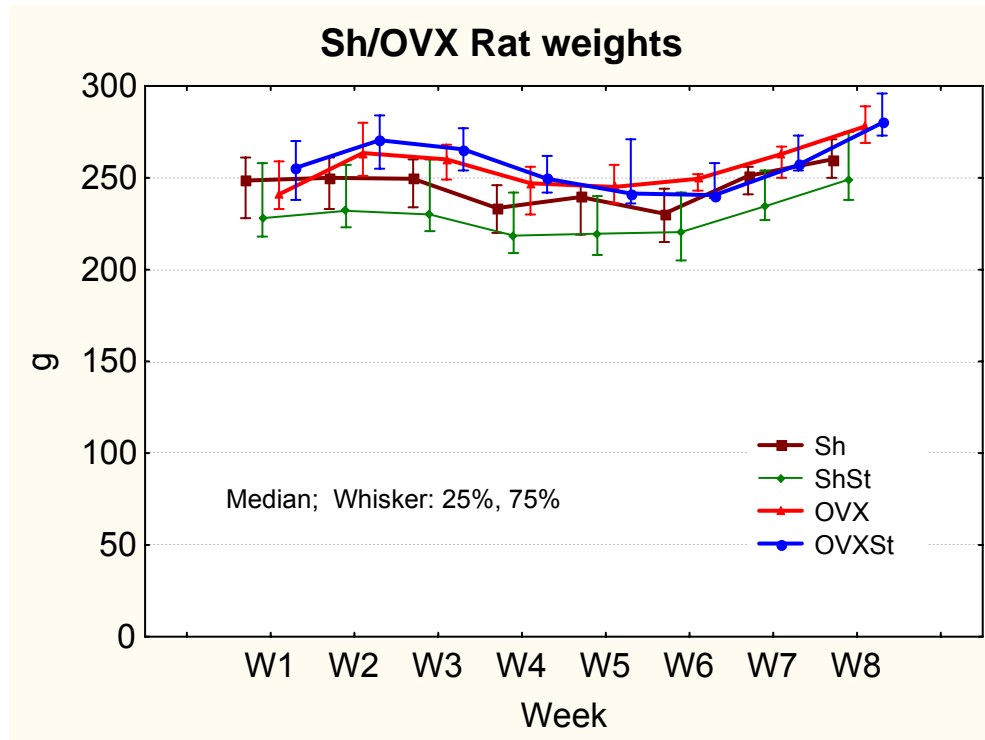
**Figure 3.1.9.** The delta value of histomorphometric parameters of bone formation in the sham-operated and ovariectomised groups.



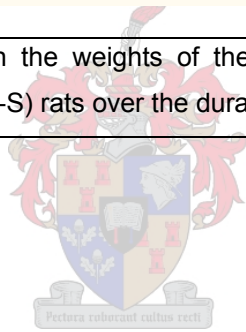
**Figure 3.1.10.** The delta value of histomorphometric parameters of bone resorption in the sham-operated and ovariectomised groups.



Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.



**Figure 3.1.11.** Changes in the weights of the sham operated (Sh, SH-S) and ovariectomised (OVX, OVX-S) rats over the duration of the study.



### **3.1.8. Discussion**

When compared to the sham-operated group (Sh), ovariectomy (OVX) resulted in a significant increase in bone formation and bone resorption. BMD was significantly decreased and this was associated with a decrease in histomorphometric bone volume. These effects of ovariectomy are expected and tend to validate the rat model used in these studies. The accuracy of our bone mineral density measurements were validated by similar results obtained at an independent center (University of Pretoria).

The average weights of the animal groups at the start of the study were comparable and the weight gain for any particular group was not significantly different from the others. Differences in body and skeletal size could not therefore explain any differences in bone mineral density. There is also no reason to believe from the histology that the statin used in this study, simvastatin, caused osteomalacia or an increase in bone marrow fat, known to result in an under-estimation of bone mineral density.

In non-ovariectomised rats (Sh, Sh-S) simvastatin had a significant effect on quantitative bone histomorphometric parameters of bone turnover. Although we had hypothesised that bone resorption would be suppressed by statins, this study surprisingly showed an increase in QBH parameters of bone resorption. Although we had anticipated that bone resorption would be primarily affected, this study also showed an increase in bone formation. Although there was a decrease in bone mineral density this did not reach statistical significance. These differences in the effect of simvastatin on ovariectomised and non-ovariectomised rats are further highlighted by the significant differences in the delta values of the parameters of bone turnover and bone mineral density in the two groups. These significant effects of simvastatin on the bone of the sham-operated rats supports the first hypothesis stated for this study. However, the finding that simvastatin increased osteoclast numbers and resorption does not support the second and third hypotheses that statins will inhibit osteoclast function and increase bone mineral density.

This seems to indicate that a mechanism other than the inhibition of prenylation may be responsible for the effect produced by simvastatin on the osteoclast.

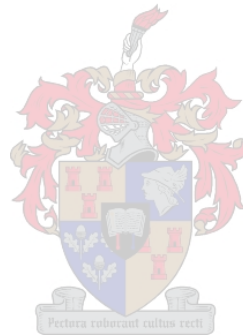
The finding that simvastatin 20mg/Kg/day increases bone formation supports the findings of other researchers that bone formation is increased. [Mundy G, Gutierrez G et al., 1998] However, our finding that simvastatin 20mg/Kg/day increases the parameters of bone resorption is somewhat at variance with the brief report of other workers. [Mundy G, Gutierrez G et al., 1998; Mundy G, Garrett R et al., 1999] These researchers primarily looked at bone formation and did not elaborate on parameters of bone resorption. Brief mention is made of a decrease in parameters of bone resorption. Later publications by the same workers also fail to report the effects of different doses of simvastatin on bone resorption. [Mundy G, Garrett R et al., 1999]

Simvastatin had no significant effect, on the QBH parameters of bone formation or resorption in the ovariectomised (OVX, OVX-S) rats of this study. Similarly the treatment with simvastatin had no effect on the bone mineral density in the ovariectomised group. This tends to disprove the last hypothesis of this study, that statins will have a greater effect in ovariectomised animals. The reasons for this are uncertain but would indicate that oestrogen could play some kind of permissive role for the effect that statins have on bone. It is also clear that simvastatin, as used in this study, was unable to prevent the decrease in bone mineral density and the increase in bone turnover produced by ovariectomy. This argues strongly against suppositions and statements that statins may be important drugs in the treatment or prevention of osteoporosis.

Although oestradiol and rFSH levels were measured to confirm the success of the ovariectomy, these levels also serve to confirm that the statins themselves did not induce a hypogonadal state. The changes induced by statins are reminiscent of oestrogen deprivation and because heterotrimeric G-protein-coupled receptors are also prenylated, there is reason to suspect that the action of trophic hormones may be interfered with. It is

Study 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

therefore unlikely simvastatin exerts its influence on bone by interfering with the action of rFSH.



### **3.1.9. Conclusions**

Although some of the findings in this study were unexpected, the following conclusions could be made:-

1. The rat model employed showed the expected densitometric and histologic changes expected following ovariectomy
2. Simvastatin increases quantitative histomorphometric parameters of both bone formation and bone resorption in non-ovariectomised rats
3. BMD was not significantly altered by simvastatin treatment although it tended to decrease (and not increase) BMD
4. There is a difference in the effect of simvastatin on histomorphometric parameters of bone turnover and bone mineral density in the presence or absence of oestrogen. Simvastatin does not have an effect on histomorphometric parameters of bone turnover or bone mineral density when oestrogen is not present
5. Simvastatin is not able to prevent the effects and consequences of ovariectomy on quantitative histomorphometric parameters of bone turnover and bone mineral density

Further studies are needed to define the role and effect of statins on bone and mineral metabolism.

Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

### **3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.**

#### **3.2.1. Background**

The results of the Study 3.1 indicated that simvastatin increases bone formation but simultaneously also stimulates osteoclastic bone resorption in sham-operated female rats but not in ovariectomised rats. This is at variance with one of the initial hypotheses, which stated that, due to the inhibitory effect of statins on protein prenylation, osteoclast function would be inhibited, and bone resorption would be suppressed, with a resultant increase in bone mineral density. Because these findings were surprising it was important to confirm these results in another study mimicking the sham-operated group but utilising intact female rats.



#### **3.2.2. Hypotheses**

The following hypothesis was made:-

- Simvastatin 20mg/Kg/day administered to intact Sprague-Dawley rats over 12 weeks will increase QBH parameters of both bone formation and resorption
- Simvastatin 20mg/Kg/day administered to intact Sprague-Dawley rats over 12 weeks will decrease BMD

#### **3.2.3. Aims of the study**

The aims of the study were therefore the following:-

1. To study the effects of simvastatin 20mg/Kg/day on BMD and QBH in intact Sprague-Dawley rats.

Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

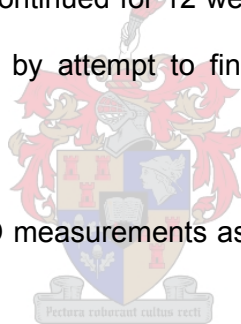
2. To compare the effects of simvastatin 20mg/Kg/day on BMD and QBH found in this study with those found in the sham-operated (Sh-S) group of Study 3.1

#### **3.2.4. Methodology**

The general rat model with the associated handling of the rats, feeding, weighing, method of drug and placebo administration, time-spaced tetracycline marking, sacrifice and harvesting of bones as described in Chapter 2.3 was utilised.

Twenty rats were randomly allocated to two groups. One group received simvastatin 20mg/Kg/day (S20) dissolved in vegetable oil as vehicle and mixed with their feeds while the remaining group received only vegetable oil as a placebo and acted as control (C). The treatment was continued for 12 weeks. The longer duration of treatment in this study was partly justified by attempt to find out whether this would make any difference to the results.

The methodology for BMD measurements as described in chapter 2.3.5 and 2.3.6 were utilised.



For the statistical analysis the results of the BMD and QBH parameters of bone formation and resorption of the S20 group were compared with those of the C group utilising the Mann-Whitney U-test. Comparisons of the effect of simvastatin on the intact rats in the present study with those of the non-ovariectomised rats in Study 3.1 were made by analysing the changes induced in the simvastatin-treated groups after treatment compared to their respective controls, the delta value. The delta values were then compared between the two studies using the Mann-Whitney U-test.

#### **3.2.5. Results**

Similar to our findings in Study 3.1, simvastatin 20mg/Kg/day produced a decreasing trend in BMD in the treated rats (S20) when compared to the untreated control

group (C) (Table 3.2.1; Fig. 3.2.1). However, this decrease in BMD did not reach statistical significance, is in agreement with the results of Study 3.1.

The static parameters of bone formation increased in the simvastatin 20mg/Kg/day treated rats (S20) compared to the untreated control group (C) (Table 3.2.1; Fig. 3.2.2). Although these increases in the formative parameters were substantial (48-110%), they did not achieve statistical significance (Table 3.2.1). Bone Formation Rate increased by 20%, but this also failed reach statistical significance ( $p = 0.15$ ). Treatment with simvastatin 20mg/Kg/day (S20) however, resulted in statistically significant increases in parameters of bone resorption when compared to the untreated control (C) (Table 3.2.1; Fig. 3.2.3.). These percent changes are qualitatively similar to those seen in the sham-operated animals (SH-S) in Study 3.1 (Fig. 3.1.4; 3.2.4).

The weights of the rats did not differ statistically between the simvastatin-treated (S20) and control (C) groups of the present study (Fig 3.2.9) (Append B 3.2). There was an average weight gain of 17.2 g between the groups over the duration of the study and the weight gain by each group, the delta value, did not differ statistically.

The descriptive statistics and statistical analyses of the data on the BMD, the descriptive statistics of the data on bone histomorphometry and the statistical analyses on the QBR data are presented in Appendices section (Append. B 3.2).



Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

### 3.2.6. Tables

**Table 3.2.1. Bone Mineral Density and Histomorphometry:**

*Simvastatin 20mg/Kg/day vs. Control.*

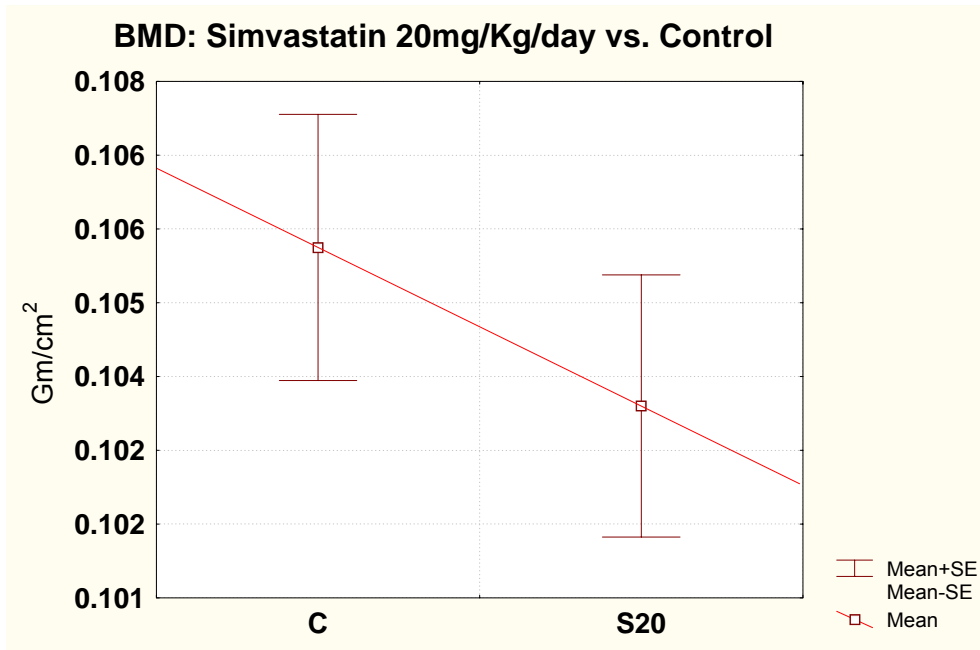
	Animal Group			
	C	S20	* %	** p
Bone Mineral Density	0.105(0.002)	0.103(0.002)	-2.04	0.4
<i>Histomorphometric parameter</i>				
Bone volume (BV/TV) (%)	23.97(1.48)	22.66(1.01)	-5	0.406
Osteoid volume (OV/BV) (%)	0.56(0.05)	0.92(0.24)	66	0.226
Osteoid volume (OV/TV) (%)	0.13(0.01)	0.20(0.04)	54	0.326
Osteoid surface (OS/BS) (%)	4.66(0.41)	6.91(1.49)	48	0.174
Osteoblast surface (Ob.S/BS) (%)	0.84(0.15)	1.75(0.42)	110	<b>0.070</b>
Osteoid thickness (O.Th) (mcm)	6.06(0.58)	6.57(0.79)	8	0.545
Eroded surface (ES/BS) (%)	7.39(0.34)	9.35(0.66)	27	<b>0.041</b>
Osteoclast surface (Oc.S/BS) (%)	0.80(0.06)	1.36(0.15)	70	<b>0.008</b>
Osteoclast number (N.Oc/TA) (/mm <sup>2</sup> )	0.07(0.01)	0.11(0.01)	75	<b>0.016</b>
Mineralizing surface (MS/BS) (%)	7.05(0.5)	7.92(0.48)	12	0.290
Mineralisation lag time (mlt) (days)	0.37(0.048)	0.33(0.04)	-10	0.705
Bone formation rate (BFR/BS) (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	17.28(1.44)	20.56(1.41)	19	0.151

Data expressed as mean (SE); C = Control; S20 = simvastatin 20mg/Kg/dy; \* = % change from Control; \*\* = vs. C.

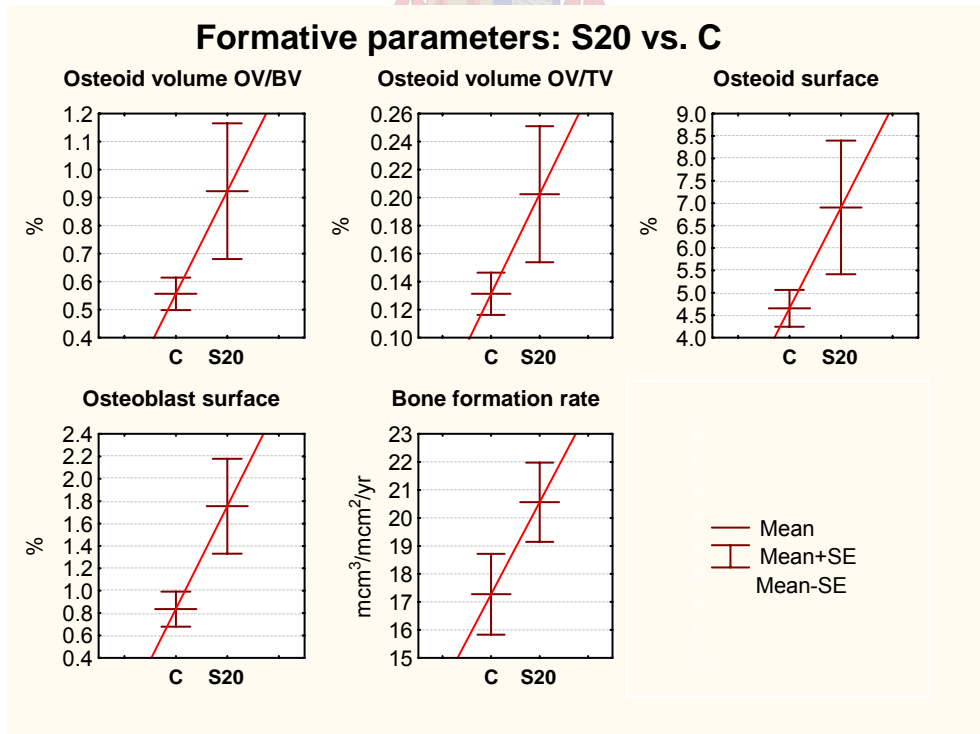


Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

### 3.2.7. Figures

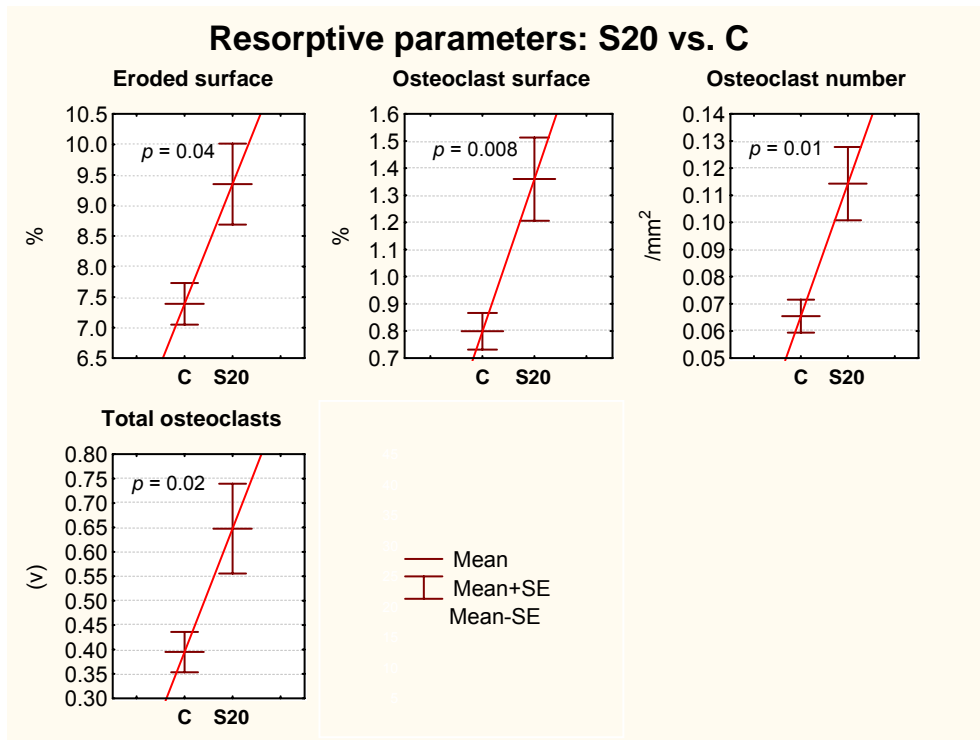


**Figure 3.2.1.** The effect of simvastatin 20mg/Kg/day on bone mineral density compared to a control group.

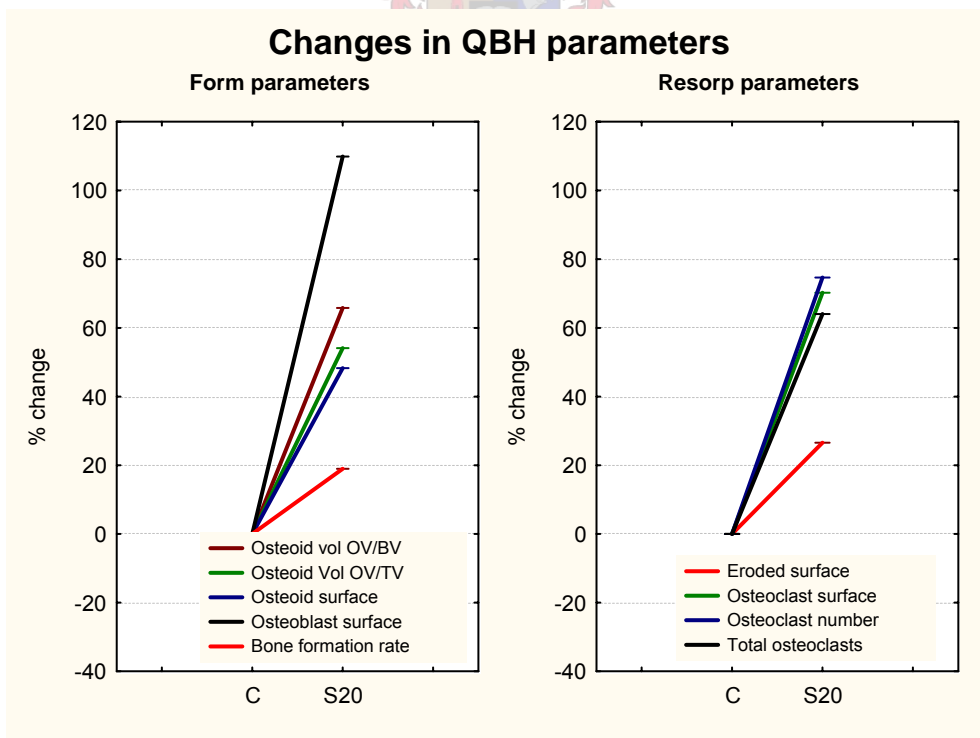


**Figure 3.2.2.** Changes induced by simvastatin 20mg/Kg/day in histomorphometric parameters of bone formation (S20) vs. the control group.

Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

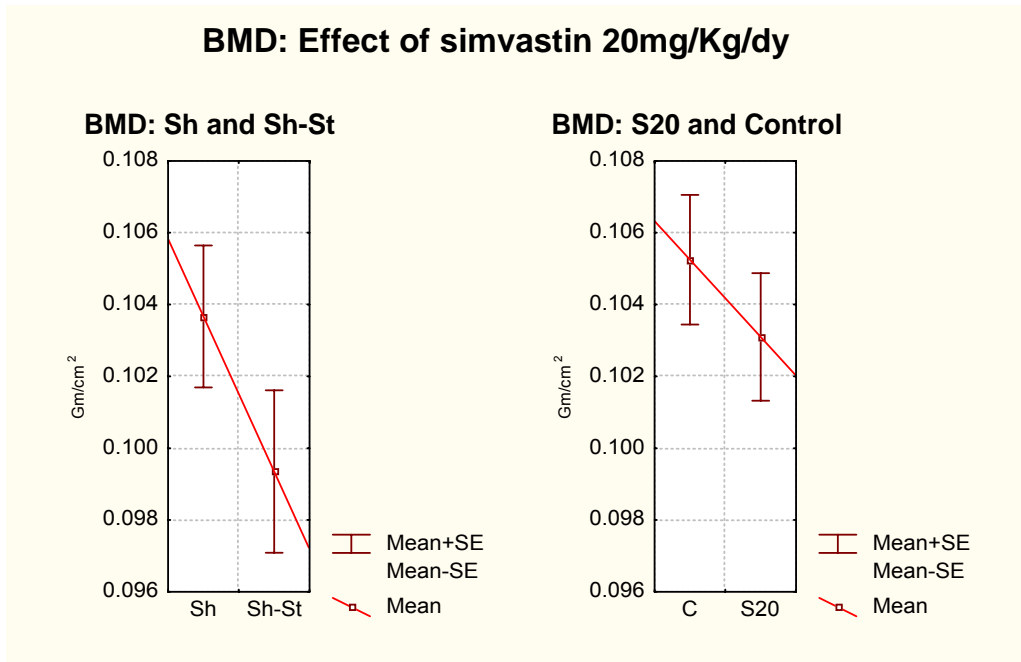


**Figure 3.2.3.** Changes induced by simvastatin 20mg/Kg/day in histomorphometric parameters of bone resorption (S20) vs. the control group.

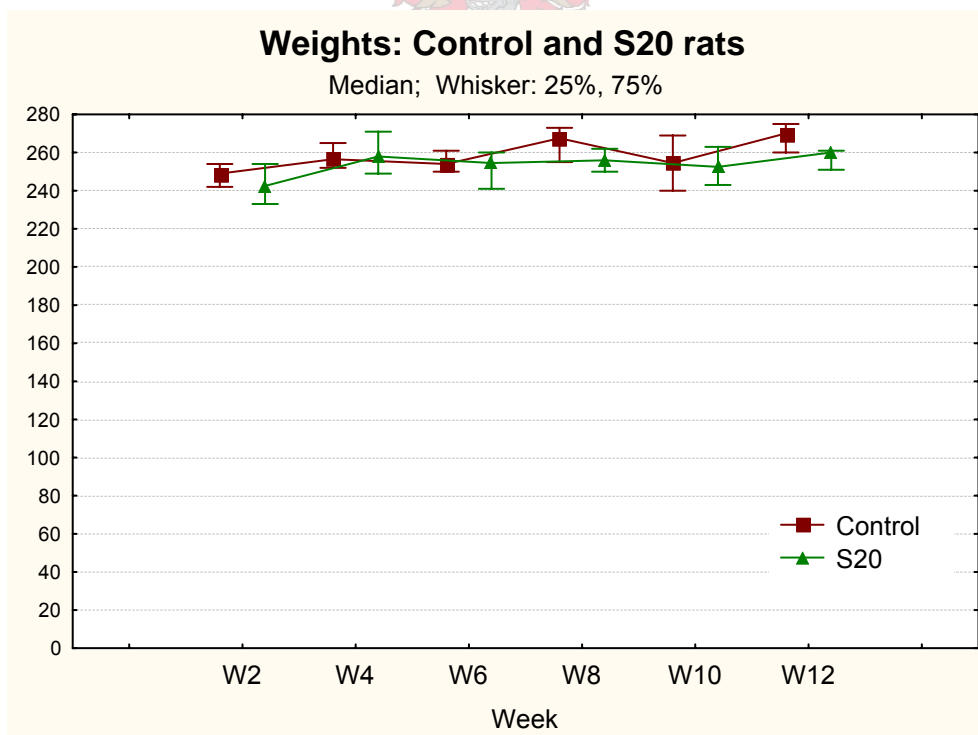


**Figure 3.2.4.** Changes in quantitative parameters of bone formation and resorption in the simvastatin-treated rats (S20) expressed as a percent change from their untreated controls (C).

Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.



**Figure 3.2.5.** BMD in the sham-operated groups and the intact rats receiving simvastatin 20mg/Kg/day - a comparison of study 3.1 and 3.2.



**Figure 3.2.6.** Changes in the weights of the Control (C) and simvastatin 20mg/Kg/day-treated rats (S20) rats over the duration of the study.

### 3.2.8. Discussion

The results of Study 3.1 indicated that osteoclast function and parameters of bone resorption were significantly increased by simvastatin 20mg/Kg/day. This was rather surprising as this was contrary to one of the hypotheses of that study which stated that osteoclast function and bone resorption would be decreased by statins - it was therefore imperative that these findings should be confirmed. The finding of study 3.1 were duplicated in this present Study 3.2 which again showed that simvastatin 20mg/Kg/day administered to non-ovariectomised rats clearly increased osteoclastic bone resorption. The fact that this has been demonstrated in two studies makes it highly likely that this is an effect of simvastatin at these doses and under these experimental conditions in non-ovariectomised rats. This study, similar to Study 3.1, also suggested that simvastatin 20mg/Kg/day increases osteoblastic bone formation, although data were less convincing.

There has been little published data on the *in vivo* effects of statins in experimental animals. Mundy and co-workers have shown that simvastatin significantly increases bone formation in rats – results not dissimilar from our own. {Mundy, Gutierrez, et al. 1998 ID: 1700}{Mundy, Garrett, et al. 1999 ID: 2629} Our finding that simvastatin 20mg/Kg/day increases bone resorption is, however, novel and unexpected. Mundy et al. [Mundy G, Garrett R et al., 1999] primarily looked at bone formation and scant detail regarding their findings on bone resorption appear in the literature. [Mundy G, Gutierrez G et al., 1998] Other researchers have also found an increase in indices of bone formation with cerivastatin. [Miller SC, Bowman BM, and Bagi C, 2001] However the magnitude and significance of these changes are not clear and it appears that the increases in parameters of bone formation were less than anticipated. Of note is that ovariectomised Sprague Dawley rats were used in these studies which contrasts with our study 3.1 where the QBH parameters of bone formation were barely affected by simvastatin 20mg/Kg/day in ovariectomised rats, changes being confined to the intact sham-operated animals. No

Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

other in vivo work has been published regarding the effects of statins on bone formation or resorption in any experimental animals..

In this study the BMD showed a decreasing trend but this was not statistically significant. These findings are similar to those found in the previous Study 3.1. This might imply that bone resorption is increased in excess of bone formation; at best the BMD indicates that bone resorption and bone formation are increased to an equal degree resulting in a BMD that is unchanged. These two studies provided no data to suggest that simvastatin increases BMD or that it prevents the deleterious effects of OVX on bone loss. There has been no published data to indicate that any other studies have been done in experimental animals to measure the effect of statins on BMD. Our studies are therefore the first which have attempted to look at this aspect of the effect of statins on bone.

### **3.2.9. Conclusions**

From the results of this study the following conclusion can be made:-

1. Simvastatin 20mg/Kg/day significantly increases QBH parameters of osteoclastic bone resorption in intact rats female rats
2. Simvastatin 20mg/Kg/day appeared to have a modest stimulatory effect on osteoblastic bone formation.
3. These effects on QBH parameters of bone turnover and BMD confirm the findings of Study 3.1.

### **3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.**

#### **3.3.1. Background**

Statins primarily reduce serum Total Cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) and to a lesser degree triglycerides. Simultaneously they also increase high-density lipoprotein-cholesterol (HDL-C). For all the statins there seems to be a dose response curve for the reduction in TC and LDL-C.[Cobos A, Jovell AJ et al., 1999] This dose response relationship is *not linear* but rather *curvilinear and plateau-shaped*. There is a lesser decrease in cholesterol for each incremental increase in the dose of the statin. If simvastatin had an effect on bone, it would therefore not be inconceivable that there would be a similar dose response curve for BMD and different QBH parameters of bone turnover.

Our own previous work only investigated the effect of a single dose of simvastatin, 20mg/Kg/day, on BMD and QBH parameters of bone turnover. These effects of statins on BMD and QBH as demonstrated in the previous two studies, Study 3.1 and 3.2, would be further validated if a dose response relationship could be demonstrated.

#### **3.3.2. Hypothesis**

The following hypotheses were thus stated:-

- There will be a dose response relationship between the dose of simvastatin and the effect on BMD, with an increasing effect on BMD with increasing doses of simvastatin following a curvilinear or plateau-shaped curve.

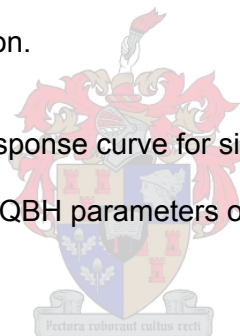
Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

- There will be a dose response relationship between the dose of simvastatin and the effect on QBH parameters of bone formation, with an increasing effect on QBH parameters of bone formation with increasing dose of simvastatin

### **3.3.3. Aims of the study**

The aims of the study were the following:-

- To examine the effect of different doses of simvastatin (20mg, 10mg, 5mg and 1mg/Kg/day) on BMD
- To examine the effect of different doses of simvastatin (20mg, 10mg, 5mg and 1mg/Kg/day) on quantitative bone histomorphometric parameters of bone formation and resorption.
- To establish a dose response curve for simvastatin and BMD, QBH parameters of bone formation and QBH parameters of bone resorption.



### **3.3.4. Methodology**

The general rat model as well as the associated handling of the rats, feeding, weighing, method of drug and placebo administration, time-spaced tetracycline marking, sacrifice and harvesting of bones as described in chapter 2.3.4 was utilised.

Fifty rats were randomly allocated to five groups of ten rats each. Four groups received active medication by way of simvastatin 20mg/Kg/day (S20), 10mg/Kg/day (S10), 5mg/Kg/day (S5) and 1mg/Kg/day (S1) respectively, dissolved in vegetable oil as vehicle, while an equivalent amount of vehicle was administered to the fifth group as placebo. Treatment was continued for 12 weeks.

The methodology for the measurement of the BMD as described in chapter 2.3.5 was utilised. The methodology for the measurement of QBH as described in chapter 2.3.6



was utilised. This standardized methodology was used for all studies thereby making these results comparable.

### **3.3.5. Results**

The BMD showed a linear decreasing trend for all the doses of simvastatin (Fig. 3.3.1). The lowest BMD and the greatest decrease in BMD was found with the lowest dose of simvastatin (1mg/Kg/day) (S1) (Table 3.3.1; Fig. 3.3.1). Statistically significant differences in BMD ( $p = 0.038$ ) when compared to control (C) were recorded for simvastatin 1mg/Kg/day (S1). A correlation between the dose of simvastatin and BMD was found ( $r^2 = 0.029$ ;  $r = 0.17$ ;  $p = 0.2$ ) and an inverse correlation between the dose of simvastatin and the degree of reduction in BMD was observed ( $r^2 = 0.03$ ;  $r = -0.19$ ;  $p = 0.2$ ) but neither reached statistical significance (Fig. 3.3.2).

The effect of the simvastatin on the QBH parameters of bone turnover varied with the different doses of simvastatin, and varying degrees of statistical significance were found (Table 3.3.1). Static as well as dynamic QBH parameters of bone formation increased with simvastatin 20mg/Kg/day (S20) but were reduced at lower doses (S1) (Fig. 3.3.3). This is additionally evident when these formative parameters are expressed as a percent change from the control value (Fig. 3.3.4); at the higher doses the QBH parameters of bone formation are increased (S20) but at lower doses these formative parameters were decreased.

The QBH parameters of bone resorption were increased by simvastatin 20mg/Kg/day (S20) (Fig. 3.3.5) and this is also evident when these parameters are expressed as a percent change from the control value (Fig. 3.3.6). With simvastatin 10mg/Kg/day and 5mg/Kg/day parameters of bone resorption decreased when compared to controls but with 1mg/Kg/day these parameters showed a percentage increase when compared to control (Figs. 3.3.5 and 3.3.6; Table 3.3.1).

The different QBH parameters of bone formation showed the same trend and moved in parallel for the different doses of simvastatin (Figs. 3.3.3 and 3.3.4). Similarly all the QBH parameters of bone resorption also showed a similar trend and moved in parallel with each other (Figs. 3.3.5 and 3.3.6). It is evident that the dose response curve for both the QBH parameters of bone formation and resorption were not linear, nor plateau-shaped. Furthermore, the dose response curve for bone formation differed qualitatively and quantitatively from the dose response curve of bone resorption (Figs. 3.3.4 and 3.3.6).

There was a statistically significant correlation between the doses of simvastatin and the different QBH parameters of bone formation (Table 3.3.2; Fig. 3.3.7). A similar statistically significant correlation between the different doses of simvastatin and the different parameters of bone resorption was evident (Table 3.3.2; Fig. 3.3.8).

The descriptive statistics and statistical analyses of the data on BMD as well as the data on the QBH parameters of bone resorption and formation, are presented in Appendices section (Appendix C 3.3).

The raw data as well as the statistical analyses and comparison of all the rat weights are presented in the Appendices section (Appendix C 3.3). The weights of the rats in the different groups were similar and there was no statistical difference in the weights of the different groups at baseline (Fig 3.3.9). There was no statistical difference in the weight gain between the different groups.

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### 3.3.6. Tables

**Table 3.3.1. Bone Mineral Density and Histomorphometry: Different Simvastatin Dosages.**

	Animal Group				
	C	S20	S10	S5	S1
<b>Bone Mineral Density</b>	0.105(0.002)	0.103(0.002)	0.1(0.002)	0.102(0.002)	0.099(0.002)
<i>Histomorphometric parameter</i>					
Bone volume (BV/TV) (%)	23.97(1.48)	22.66(1.01)	20.41(1.27)	22.97(0.87)	21.04(1.62)
Osteoid volume (OV/BV) (%)	0.56(0.05)	0.92(0.24)	0.48(0.15)	0.42(0.06)	0.43(0.14)
Osteoid volume (OV/TV) (%)	0.13(0.01)	0.20(0.04)	0.09(0.02)	0.10(0.01)	0.08(0.02)
Osteoid surface (OS/BS) (%)	4.66(0.41)	6.91(1.49)	3.96(1.18)	3.68(0.5)	2.86(1.13)
Osteoblast surface (Ob.S/BS) (%)	0.84(0.15)	1.75(0.42)	0.44(0.17)	0.67(0.11)	0.53(0.18)
Osteoid thickness (O.Th) (mcm)	6.06(0.58)	6.57(0.79)	6.55(1.34)	6.46(1.06)	8.51(1.26)
Eroded surface (ES/BS) (%)	7.39(0.34)	9.35(0.66)	6.95(1.22)	5.30(0.36)	6.78(0.73)
Osteoclast surface (Oc.S/BS) (%)	0.80(0.06)	1.36(0.15)	0.74(0.21)	0.56(0.12)	0.97(0.14)
Osteoclast number (N.Oc/TA) (/mm <sup>2</sup> )	0.07(0.01)	0.11(0.01)	0.07(0.02)	0.06(0.01)	0.10(0.01)
Mineralizing surface (MS/BS) (%)	7.05(0.5)	7.92(0.48)	5.42(1.22)	4.54(0.25)	5.04(1.01)
Mineralisation lag time (mlt) (days)	0.37(0.048)	0.33(0.04)	0.73(0.18)	0.40(0.07)	1.22(0.27)
Bone formation rate (BFR/BS) (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	17.28(1.44)	20.56(1.41)	12.14(2.65)	16.71(1.41)	9.69(2.4)

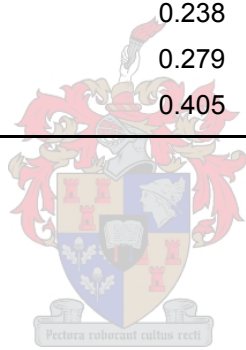
	% change of parameter and significance							
	S20		S10		S5		S1	
	* %	** p	* %	** p	* %	** p	* %	** p
<b>Bone Mineral Density</b>	-2.04	0.4	-4.9	0.085	-2.7	0.338	-5.84	0.0386
<i>Histomorphometric parameter</i>								
Bone volume (BV/TV) (%)	-5	0.406	-15	0.070	-4	0.450	-12	0.191
Osteoid volume (OV/BV) (%)	66	0.226	-13	0.112	-24	0.151	-23	0.060
Osteoid volume (OV/TV) (%)	54	0.326	-29	0.096	-27	0.089	-40	0.033
Osteoid surface (OS/BS) (%)	48	0.174	-15	0.070	-21	0.096	-39	0.007
Osteoblast surface (Ob.S/BS) (%)	110	0.070	-47	0.016	-20	0.496	-37	0.142
Osteoid thickness (O.Th) (mcm)	8	0.545	8	0.850	7	1.000	40	0.079
Eroded surface (ES/BS) (%)	27	0.041	-6	0.151	-28	0.002	-8	0.327
Osteoclast surface (Oc.S/BS) (%)	70	0.008	-7	0.070	-30	0.034	22	0.369
Osteoclast number (N.Oc/TA) (/mm <sup>2</sup> )	75	0.016	13	0.450	-14	0.257	49	0.165
Mineralizing surface (MS/BS) (%)	12	0.290	-23	0.019	-36	0.002	-29	0.014
Mineralisation lag time (mlt) (days)	-10	0.705	98	0.326	9	0.940	230	0.007
Bone formation rate (BFR/BS) (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	19	0.151	-30	0.013	-3	0.705	-44	0.022

Data expressed as mean (SE); C = Control; S20 = simvastatin 20mg/Kg/dy; S10 = simvastatin 10mg/Kg/dy; S5 = simvastatin 5mg/Kg/dy; S1 = simvastatin 1mg/Kg/dy; \* = % change from Control; \*\* = vs. C.

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

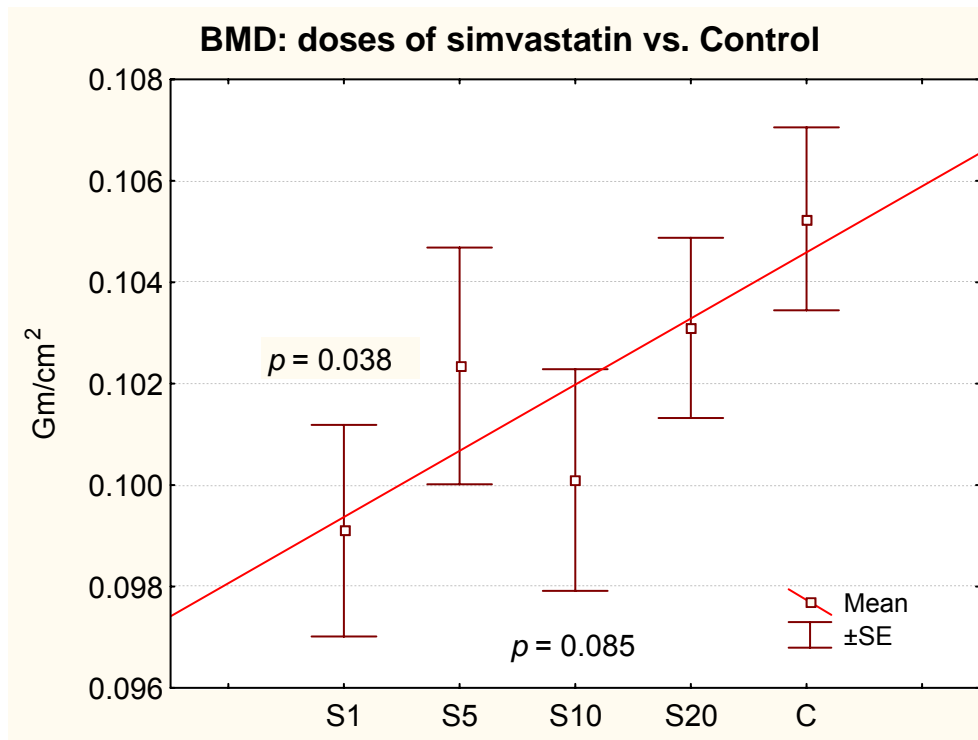
**Table 3.3.2.** Correlation between different dosages of simvastatin and quantitative histomorphometric parameters.

<b>Formative Parameters</b>				
	<b>r(X,Y)</b>	<b>r<sup>2</sup></b>	<b>t</b>	<b>p</b>
Osteoid Volume (OV/BV)	0.369	0.136	2.415	0.0208
Osteoid Volume (OV/TV)	0.449	0.201	3.054	0.004
Osteoid Surface	0.403	0.162	2.676	0.011
Osteoblast Surface	0.490	0.240	3.423	0.001
Bone Formation Rate	0.445	0.198	3.023	0.004
<b>Resorptive Parameters</b>				
	<b>r(X,Y)</b>	<b>r<sup>2</sup></b>	<b>t</b>	<b>p</b>
Eroded Surfaces	0.438	0.191	2.964	0.005
Osteoclast Surface	0.362	0.131	2.367	0.023
Osteoclast Number	0.238	0.056	1.492	0.144
Total Osteoclasts	0.279	0.078	1.767	0.085
Bone Osteoclast Interface	0.405	0.164	2.697	0.010

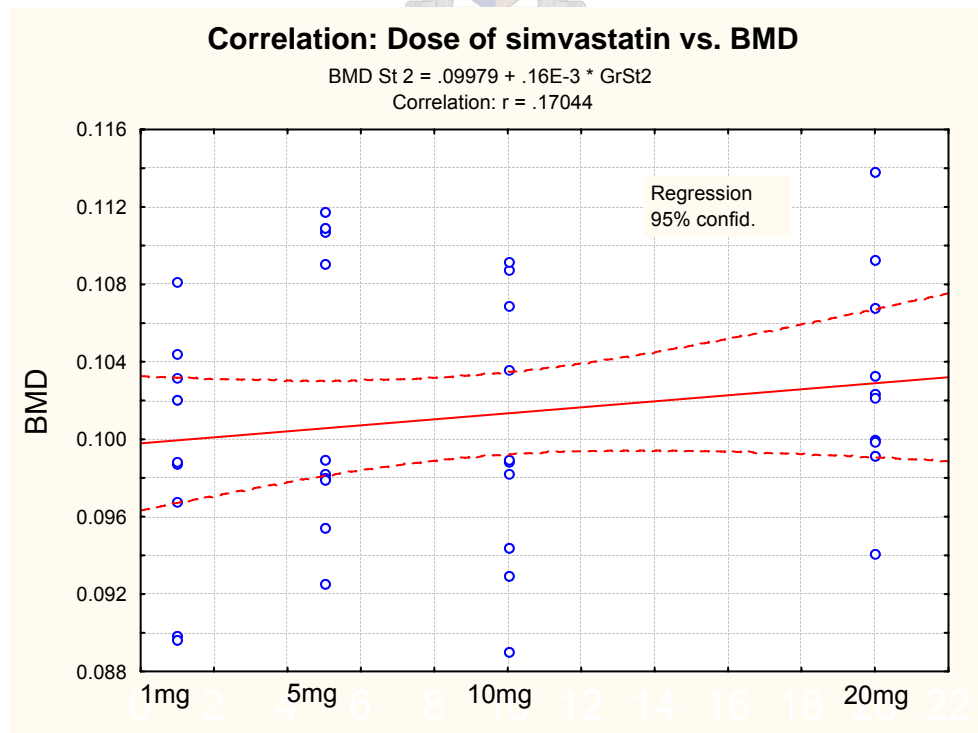


Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### 3.3.7. Figures

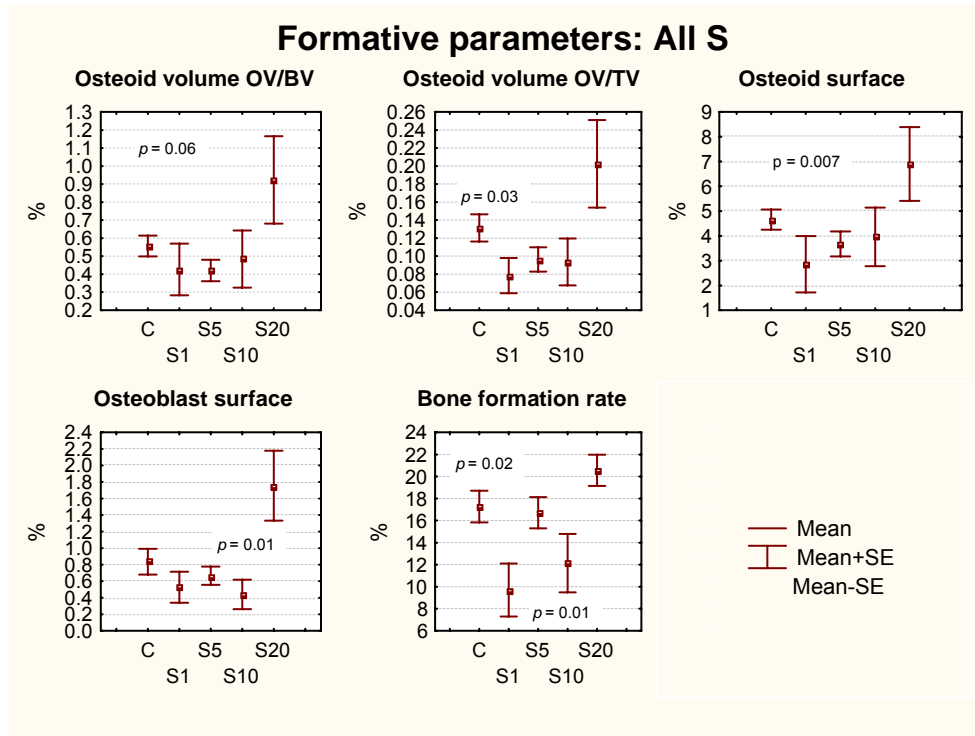


**Figure 3.3.1.** The effect of simvastatin 20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day on bone mineral density compared to a control group. \* = vs. C.

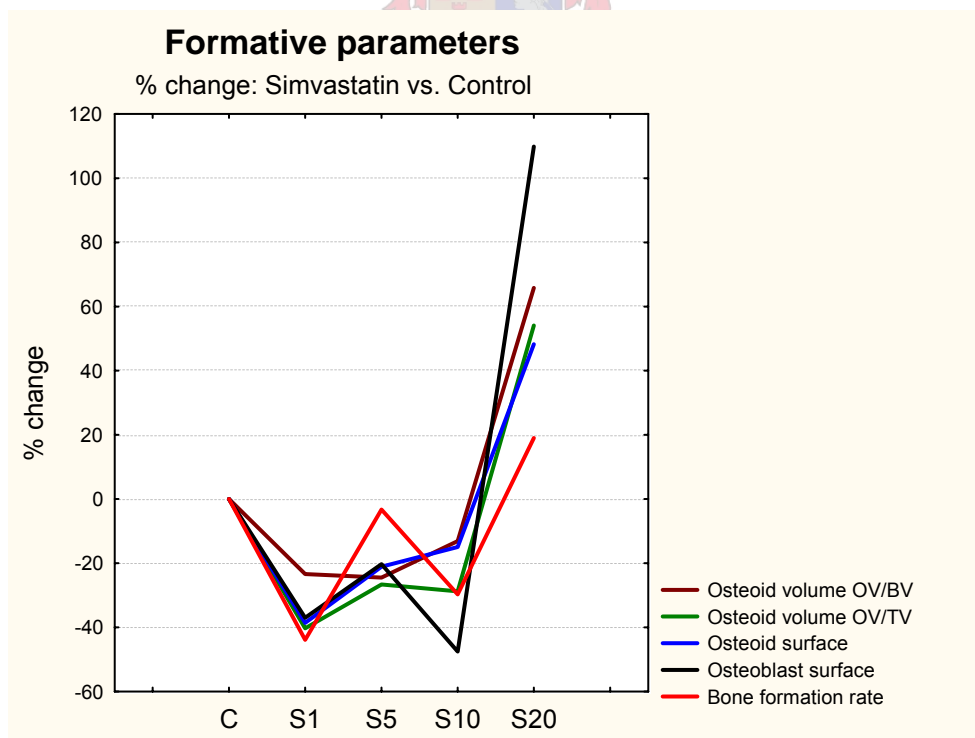


**Figure 3.3.2.** Correlation between simvastatin dose and BMD.

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

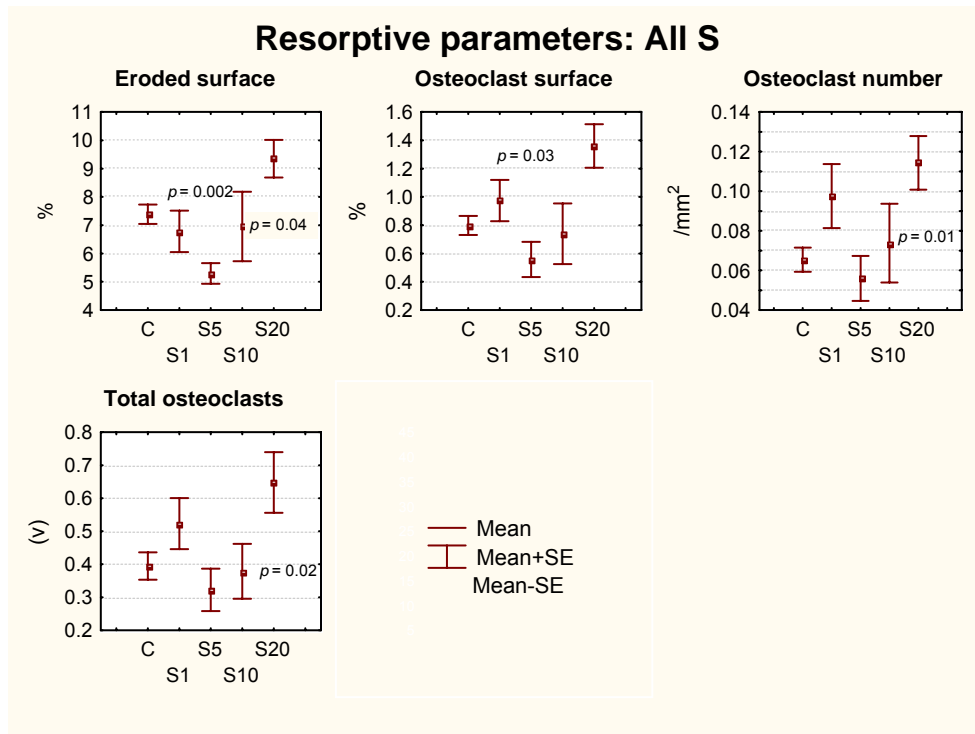


**Figure 3.3.3.** The effect of different doses of simvastatin on QBH parameters of bone formation. \* = vs. C.

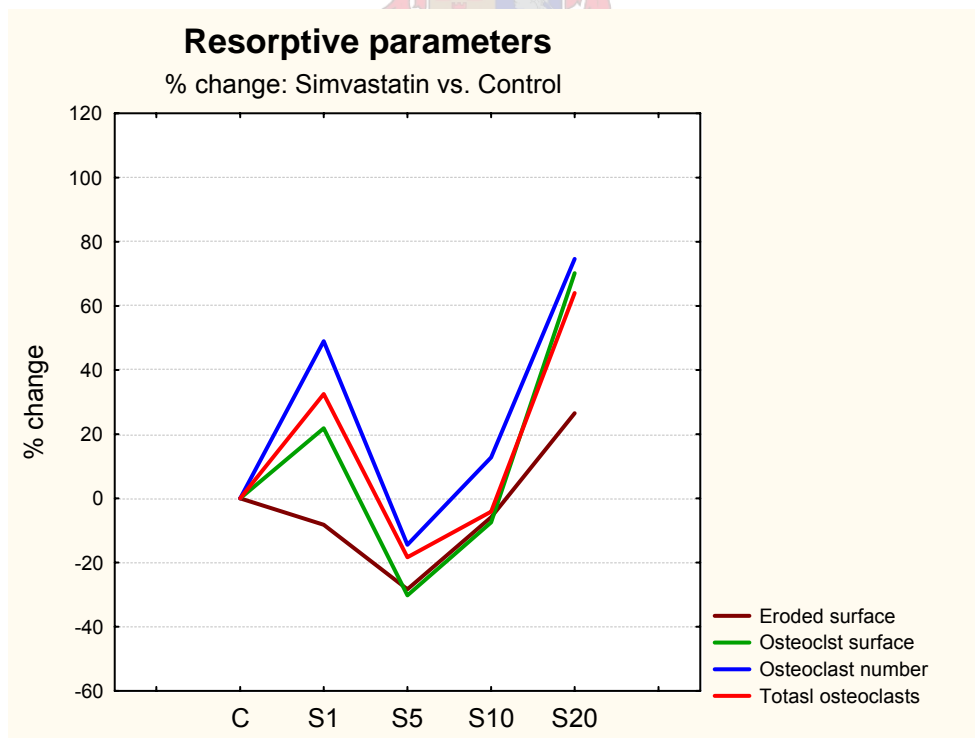


**Figure 3.3.4.** The effect of different doses of simvastatin on the percent changes in the QBH parameters of bone formation.

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

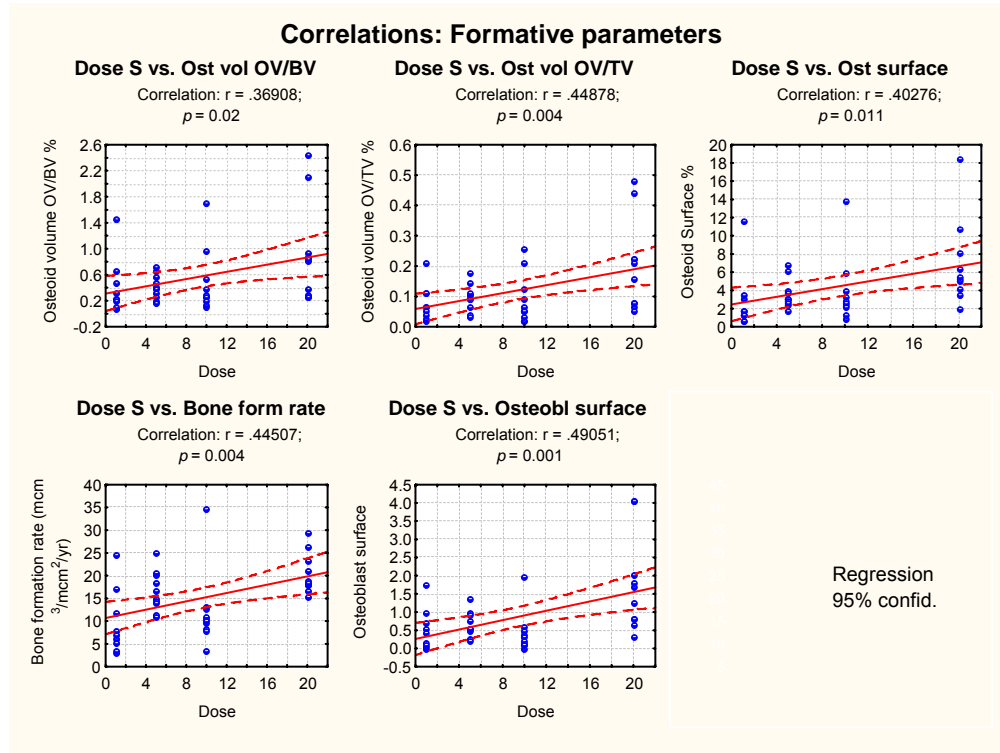


**Figure 3.3.5.** The effect of different doses of simvastatin on QBH parameters of bone resorption. \* = vs. C.

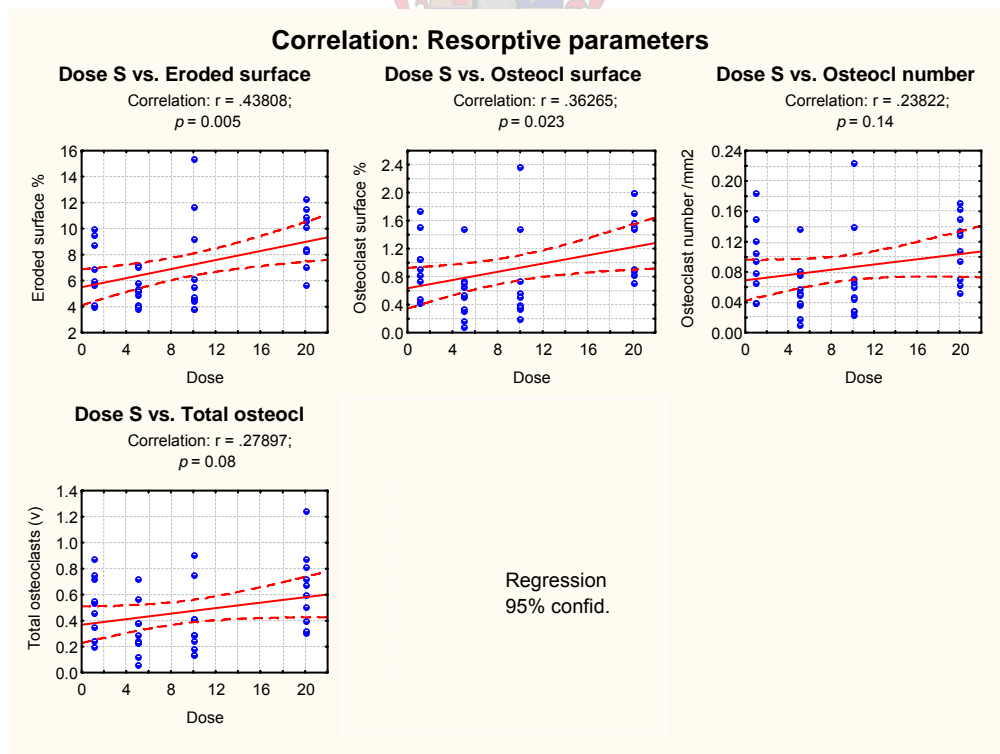


**Figure 3.3.6.** The effect of different doses of simvastatin on the percent changes in QBH parameters of bone resorption.

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.



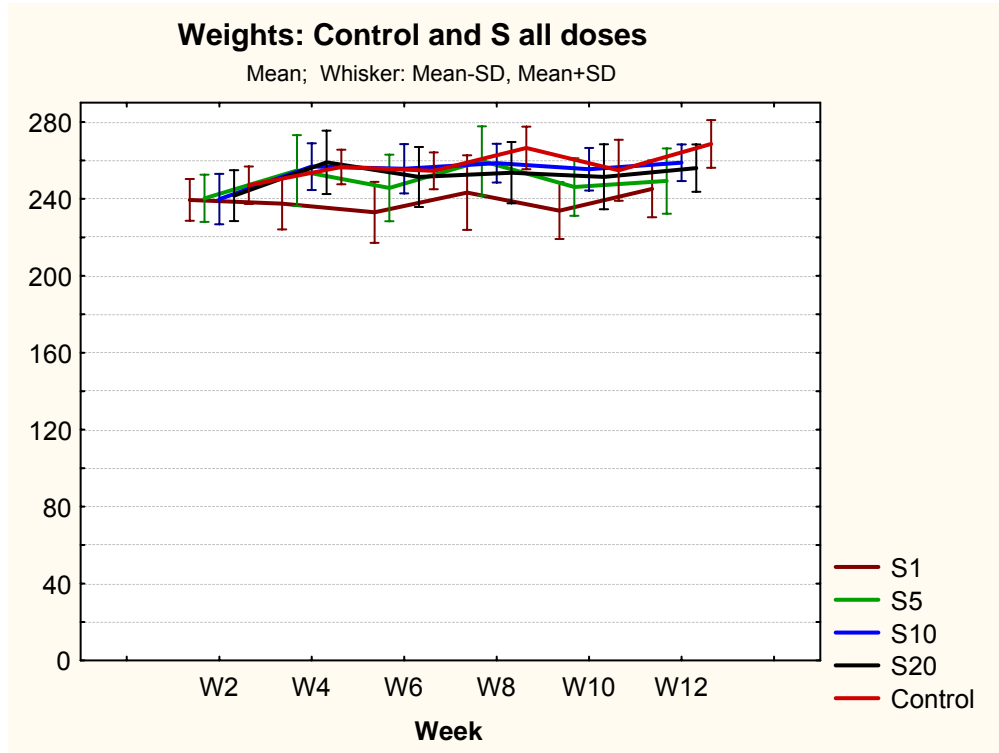
**Figure 3.3.7.** Correlation between dose of simvastatin and QBH parameters of bone formation.



**Figure 3.3.8.** Correlation between dose of simvastatin and QBH parameters of bone resorption.



Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.



**Figure 3.3.9.** Weights of the different simvastatin dose groups and control over the duration of the study.



### **3.3.8. Discussion**

It has been demonstrated in this study that as the dose of simvastatin changes so do the QBH parameters of bone turnover. A significant correlation between the dose of simvastatin and the QBH parameters of bone formation has been demonstrated in this study and accordingly a dose response curve has been constructed. At an high dose of 20mg/Kg/day simvastatin stimulated osteoblastic activity and increased bone formation. However, at lower doses (1mg/Kg/day) this effect was not seen and instead osteoblast activity was inhibited and formation was decreased.

Similarly a significant correlation between the dose of simvastatin and QBH parameters of bone resorption has been demonstrated and accordingly it has been possible to construct a dose response curve. At the highest dose of simvastatin, 20mg/Kg/day, osteoclasts are stimulated and bone resorption increased. At the lower doses of simvastatin (10mg/Kg/day and 5mg/Kg/day) osteoclasts are inhibited and bone resorption is decreased. At lower doses still, simvastatin 1mg/Kg/day again increases bone resorption. Accordingly a U-shaped dose response curve is evident.

It is clear, particularly when looking at the percent changes in the QBH parameters of bone formation, that all the static and dynamic parameters move in the same direction and in a parallel fashion. This is also seen with the parameters of bone resorption when expressed as percent changes from baseline; all the parameters move in concert in the same direction and in a parallel fashion.

If the dose response curves of the QBH parameters of bone formation are compared with the dose response curves of the resorptive parameters it is clearly evident that these dose response curves are not the same. This finding may have important implications for explaining the findings seen on the BMD measurements.

It is apparent that the effects of simvastatin on the QBH parameters of bone resorption and formation at high doses differ from those seen at low doses. Clearly these dose-response curves are also not complete. The simvastatin needs to be investigated at ever smaller doses till the smallest dose is found where there is no effect on any of the QBH parameters of bone formation or resorption. If the dose response curve of the formative parameters is extended to the left to a point where they reach the nil value on the Y-axis, (Response axis), then it becomes evident that there is a biphasic response to simvastatin. Bone formation is stimulated at high doses and at lower doses this does not simply decrease to a nil value where no response is seen but the response goes below the baseline nil value, i.e. bone formation is decreased at lower doses. For bone resorption the dose-response is somewhat more complex but also shows a biphasic response.

The underlying mechanism for this biphasic response is not immediately clear. The one possible explanation for this phenomenon would be that there could be two or more signaling pathways involved in mediating the response to simvastatin and the final effect on the osteoblast or osteoclast. If the dose response curves for these signalling pathways differ then it may happen that at a lower dose only a single signalling pathway may be stimulated whereas at a higher dose more than one pathway may be stimulated with a resultant differing effect. However, this is purely supposition and there is no data to support this.

The different doses of simvastatin have a significant effect on BMD (ANOVA). There is a linear relationship between the doses of simvastatin and BMD.

There is an indirect correlation between the dose of simvastatin and the BMD with the highest and lowest doses of simvastatin associated with the lost and highest BMD respectively. There is an inverse relationship between the dose of simvastatin and the amount of reduction in the BMD i.e. the largest decreases were seen with the smallest

dose of simvastatin while little change was seen with the largest doses of simvastatin. This effect is rather unconventional and unexpected. However, this kind of response is possible if there are two process present which together determine the BMD reading and if the dose response curves of these two processes differ. And there are indeed two processes that determine the BMD, namely bone formation and bone resorption. If the dose response curves of bone formation and resorption were to differ then it could happen that bone resorption is stimulated at low doses of simvastatin and that bone formation is only stimulated at higher doses of simvastatin. This would mean that at low doses of simvastatin, only resorption would be stimulated, with a resultant decrease in BMD. At higher doses resorption and formation would be stimulated, the two processes would tend to balance one another and there would be little change in the BMD. Differing dose response curves for bone resorption and bone formation have been demonstrated in this study and the above could be a plausible explanation for the BMD findings in this study.

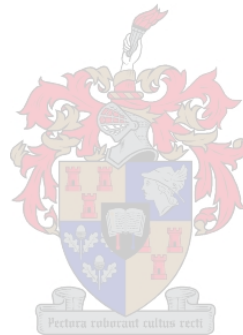
### **3.3.9. Conclusions**

The study allowed the following conclusions to be made:-

1. Simvastatin has an influence on BMD and small doses of simvastatin are associated with a reduction in the BMD.
2. The effect of simvastatin on QBH parameters of bone turnover differs according to dose of simvastatin used
3. The dose-response curve for simvastatin on QBH parameters of bone formation differs from the dose-response curve for QBH parameters of bone resorption.
4. At high simvastatin doses of 20mg/Kg/day both bone formation and bone resorption are stimulated with little associated effect in the BMD.

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

5. At the low dose of simvastatin 1 mg/kg/day bone formation is suppressed whereas bone resorption is mildly stimulated, resulting in a marked decrease in BMD.



### **3.4 The effect atorvastatin 2.5mg/Kg/day and pravastatin 10mg/Kg/day administered for 12 weeks, on bone mineral density in intact female Sprague-Dawley rats.**

#### **3.4.1. Background**

In studies 3.1, 3.2 and 3.3 it was established that simvastatin, at various doses, had an effect on bone metabolism. The effect of simvastatin on bone formation and resorption differed according to the dosage of simvastatin. The statins all inhibit the rate-limiting enzyme of the cholesterol synthetic pathway, hydroxymethylglutaryl CoA reductase. Research done by others indicates that the effect of statins on bone involves the inhibition of prenylation via the reduction of substrates, an effect shared by all the statins. [Coxon FP, Benford HL et al., 1998; Frith JC, Armour KJ et al., 2001; Garret IR, Esparza J et al., 2000; Garret IR, Gutierrez G et al., 2001; Guijarro C, Blanco-Colio LM et al., 1998; Hughes AD, 1996; Laufs U and Liao JK, 2000] There is little reason to believe that statins other than simvastatin will not also have a similar on the inhibition of prenylation and therefore have an effect on bone metabolism.

Statins have other effects and it is commonly assumed that all the effects of one statin are automatically shared by all the other statins via a class effect. The statins simvastatin, atorvastatin and pravastatin differ from each other in major ways and it would not be unreasonable to suspect that effect that they might have on bone will also differ.

There are reasons enough to expect that there will not be a class-effect for many of the effects attributed to statins. The chemical formulae of the statins differ markedly and are often divided into the "natural" and "synthetic" statins" where simvastatin and pravastatin are classed as "natural" and atorvastatin classed as "synthetic". [Rosenson RS and Tangney CC, 1998] These dissimilarities may affect the way that they bind to target molecules, and engender different properties to the various statins.

The metabolism of the statins differ to a major degree. Atorvastatin, lovastatin, simvastatin and cerivastatin are primarily metabolised by cytochrome CYP3A4 [Corsini A, Bellosta S et al., 1999a; Kantola T, Kivisto KT, and Neuvonen PJ, 1998] whereas the metabolism of fluvastatin utilises a different cytochrome. Pravastatin is metabolised to a large degree in the stomach and pravastatin does not make use of the cytochrome P450 systems for its metabolism. This can have important implications regarding interactions with drugs that also use of these P450 systems. [Azie NE, Brater DC et al., 1998] Atorvastatin is eliminated mainly by the liver whereas pravastatin is eliminated by other mechanisms.

The lipid solubility of the statins differs. Pravastatin is hydrophilic whereas all the others are lipophilic. Consequently, based on lipid solubility, the ability of different statins to cross membranes will differ. Accordingly, the intracellular effect of the statins will also differ when delivered from the exterior of the cell. For these reasons, the first pass extraction by the liver after oral administration will also differ; for simvastatin this exceeds 90% [Vickers S, Duncan CA et al., 1990; Desager JP and Horsmans Y, 1996] and for pravastatin it is in the order of 60% [Komai T, Kawai K et al., 1992] and as a result the amount of statin which reaches peripheral target cells other than the liver will be different. Relatively little active simvastatin reaches the systemic circulation where it can consequently affect peripheral tissues such as bone cells. On the other hand more pravastatin reaches the systemic circulation and relatively more pravastatin is therefore available to have an effect on peripheral cells such as bone cells. [Germershausen JI, Hunt VM et al., 1989; Hatanaka T, 2000].

Compared to other statins, pravastatin has more sterol-inhibitory action in the liver than in peripheral tissues such the spleen and testis despite having similar drug levels in those particular tissues. [Koga T, Fukuda K et al., 1992] However, because of its hydrophilicity, pravastatin does not cross membranes readily and much of the pravastatin that reaches peripheral tissues is present extracellularly, compared to other lipophilic

statins which are located intracellularly. [Koga T, Fukuda K et al., 1992] This inability of pravastatin to cross membranes easily might therefore offset the lower first pass effect and consequent higher plasma levels seen with pravastatin. Presumably this lesser action on peripheral tissues and cells might also apply to bone. The first pass extraction by liver has other important implications. Statins delivered by dermal application will bypass the first pass extraction by the liver and a greater proportion of the drug will reach the bone.

The above factors will all affect the arterial concentration of the different statins and therefore also the concentration of drug that reaches the blood bone interface. We have already shown that the effect of simvastatin on bone is dependant on the dosage of the drug. The differing relative arterial concentrations of the statin will therefore also influence to what degree they will affect bone metabolism.

The half-life of most statins is in the order of 2 hours whereas atorvastatin has a half-life exceeding 18 hours. [Posvar EL, Radulovic LL et al., 1996; Cilla DD, Whitfield LR et al., 1996; Desager JP and Horsmans Y, 1996] The administration of atorvastatin therefore results in continuously raised blood levels of the drug during the course of a 24 hour day with no dips in the drugs levels; consequently cells are continuously exposed to the effect of the statin. This may be one of the reasons for the cholesterol-lowering potency of the drug. The other statins have therapeutic levels for only part of the day and there are long periods when cells are not under the influence of these drugs. The use of atorvastatin therefore amounts to continuous dosing compared to micro-intermittent dosing with the use of the other statins. Differences in the effect of parathyroid hormone on bone have been noted when continuous dosing is compared to intermittent dosing. Continuous dosing with PTH results in osteopenia whereas intermittent dosing with PTH is associated with an increase in BMD. [Masiukiewicz US and Insogna KL, 1998] Bearing in mind the number of important signalling systems that are impinged upon by the statins, particularly those utilising prenylated proteins which play a pivotal role in cell growth,



differentiation and activation of cells, these differences in half-life may have important consequences in different organ systems.

It can therefore be expected that other statins such as atorvastatin and pravastatin will also have an effect on bone health and which might differ from that seen with simvastatin.

### **3.4.2. Hypothesis**

Based on differences in the pharmacokinetics and metabolism of the statins the following hypothesis was stated:-

- The effect of the long-acting atorvastatin on BMD will differ from that seen with the short-acting simvastatin.
- The effect of the hydrophilic pravastatin on BMD will differ from that seen with the lipophilic simvastatin.



### **3.4.3. Aims of the study**

Accordingly the aims of the study were the following:-

- To investigate the effect of atorvastatin 2.5mg/Kg/day and pravastatin 10mg/Kg/day on BMD compared to controls

### **3.4.4. Methodology.**

The general rat model with the associated handling of the rats, feeding, weighing, method of drug and placebo administration, sacrifice and harvesting of bones as described in chapter 2.3.4 was utilised.

Thirty rats were randomly allocated to three groups of ten rats each. One group received atorvastatin 2,5mg/Kg/day (A) dissolved in vegetable oil vehicle, another

received pravastatin 10mg/Kg/day (P) in a similar fashion while the remaining group received only vehicle as placebo and acted as control (C). The treatment was continued for 12 weeks.

The dose of atorvastatin and pravastatin chosen, (2.5mg/Kg/day and 10mg/Kg/day respectively) was that which was expected to produce the same cholesterol-lowering effect as simvastatin 5mg/Kg/day. There is no data on the comparative doses of statin which produce the same cholesterol-lowering effect in rats. The doses used in this study were therefore chosen because of the cholesterol-lowering comparisons and recommendations between different statins in humans [Illingworth DR and Tobert JA, 1994]. From these comparisons, it is generally accepted that simvastatin 20mg, pravastatin 40mg and atorvastatin 10mg per day have the same cholesterol-lowering effect in humans.

The methodology for bone mineral density measurements as described in chapter 2.3.5 was utilised.



### **3.4.5. Results**

Both atorvastatin 2.5mg/Kg/day (A) and pravastatin 10mg/Kg/day (P) administered for 12 weeks produced a highly significant reduction in BMD when compared to the control group (C) (Fig. 3.4.1; Table 3.4.1).

The descriptive statistics and statistical analyses of the BMD data, are presented in Appendices section (Append. 3.4).

There was an average weight gain of 25g for all the groups and the weight gain by each group did not differ statistically.

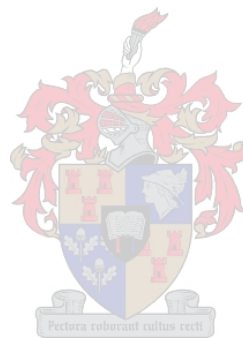
Study 3.4 The effect atorvastatin 2.5mg/Kg/day and pravastatin 10mg/Kg/day administered for 12 weeks, on bone mineral density in intact female Sprague-Dawley rats.

### 3.4.6. Tables

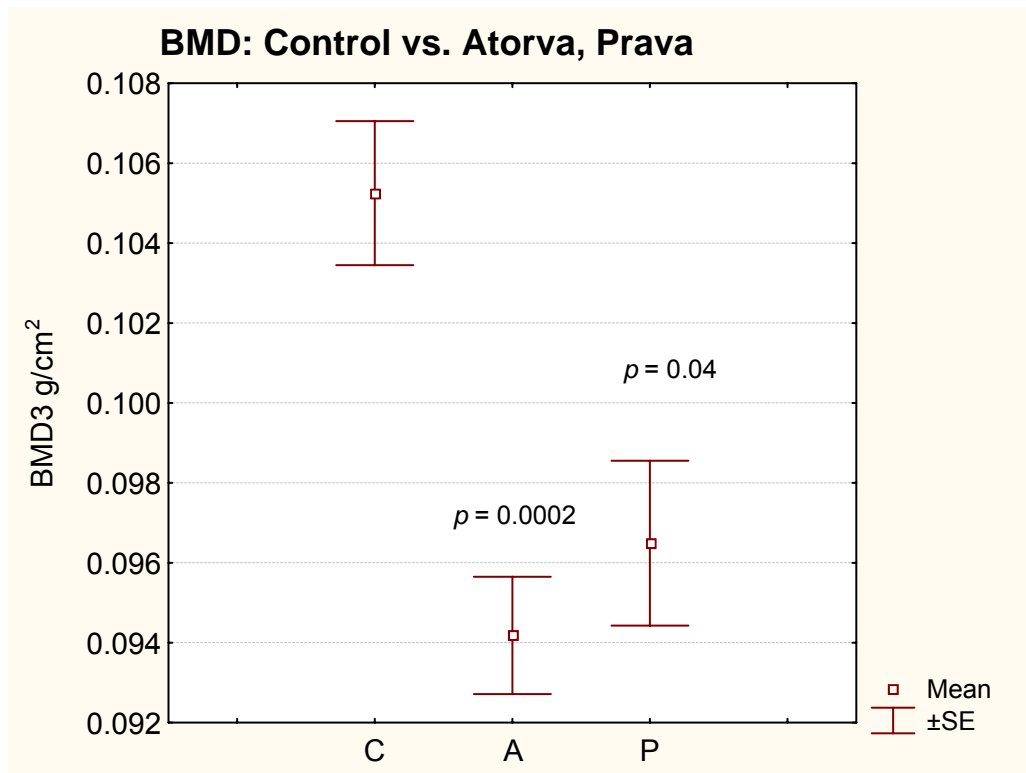
**Table 3.5.1. Bone Mineral Density: Atorvastatin, Pravastatin vs. Control**

	Animal Group						
	Control		Atorvatstin			Pravastatin	
	* %	** p	* %	** p		* %	** p
<b>Bone Mineral Density</b>	0.1053	0.0942	-10.5	0.0002	0.0965	-8.3	0.005

Data expressed as mean (SE); \* = % change from Control; \*\* = vs. Control.



### 3.4.7. Figures



**Figure 3.4.1.** The effect of atorvastatin 2.5mg/Kg/day (A) and pravastatin 10mg/Kg/day (P) on BMD compared to the control group (C).



Study 3.4 The effect atorvastatin 2.5mg/Kg/day and pravastatin 10mg/Kg/day administered for 12 weeks, on bone mineral density in intact female Sprague-Dawley rats.

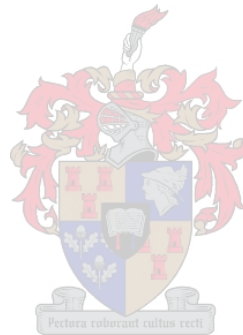
### **3.4.8. Discussion**

The discussion of these results will follow in Chapter 4.

### **3.4.9. Conclusions**

The following conclusion were made after the completion of the study:-

- Both atorvastatin 2.5mg/Kg/day and pravastatin produced marked and significant reductions in BMD compared to the untreated controls



## Chapter 4: Discussion

### 4.1. Validity of the rat model and the study results.

These studies have shown that simvastatin 1mg/Kg/day, atorvastatin 2,5mg/Kg/day and pravastatin 10mg/Kg/day decrease BMD, and that statins have a variable effect on QBH parameters of bone formation and resorption. However, some of these findings are in conflict with hypotheses stated at the start of the studies. Could these results have been the result of a methodological error?

The studies were all performed in a standard fashion and under identical conditions. One rat in the S1 group died at nine weeks of unknown causes. No other illnesses amongst the rats were noted, they were otherwise healthy and there were no other deaths. The rats were randomly allocated between the different groups and there were no significant differences in the weights of the different groups. The rat feeds were adjusted to keep the weight of the all groups constant but despite this there was a modest weight gain by all the rat groups. The weight gain in the different groups was similar and there was no statistical difference in the weight gain between the different groups.

The BMD of the same specimens was measured at two different accredited academic centres. The results obtained at these two centres were identical and did not differ statistically from each other. The BMDs at our centre were measured by a single experienced technologist who was blinded to the treatment groups of the rats. Similarly the technologist who performed the QBH was also blinded to the treatment group of the rats. Therefore bias at this level does not seem probable.

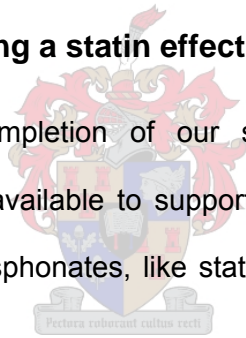
Except for the treatment given, the methodology for all the groups was identical in all the studies. The results of the QBH and BMD obtained from the ovariectomised (OVX) and sham-operated rats (Sh) were also consistent with the expected findings of an ovariectomy model. The OVX rats showed the expected large and significant decrease in

BMD compared to the Sh rats. Additionally the OVX rats also showed the anticipated increase of bone turnover, with an increase in both bone formation and resorption compared to the Sh group supporting the validity of the model. Furthermore the effects of simvastatin 20mg/Kg/day on BMD and QBH in the sham-operated rats (Sh-S) were independently confirmed in a separate later study utilising simvastatin 20mg/Kg/day in intact rats (S20).

From the histology there is also no reason to believe that the statins used in our study caused osteomalacia or an increase in bone marrow fat, known to result in an underestimation of BMD. Our data showing that the administration of statins was associated with a reduced BMD are convincing.

## **4.2. Additional data supporting a statin effect on bones.**

Since the start and completion of our studies, further basic science and experimental data has become available to support the notion that statins will have an effect on bones and that bisphosphonates, like statins, inhibit prenylation [Luckman SP, Hughes DE et al., 1998]:-



### **4.2.1. Bisphosphonates, prenylation and the effects on osteoclasts**

There is evidence that the ultimate target for bisphosphonates is the osteoclast and that these drugs inhibit the activity of, and cause apoptosis of, osteoclasts, and also inhibit osteoclastogenesis. [Rodan GA, 1998; Luckman SP, Coxon FP et al., 1998a] Indeed, the nitrogen-containing bisphosphonate, alendronate, inhibits the cholesterol synthetic pathway via inhibition of isopentenyl pyrophosphate isomerase/farnesylpyrophosphate synthase, an enzyme two steps distal to HMG-CoA reductase. [van Beek ER, Pieterman E et al., 1999; Bergstrom JD, Bostedor RG et al., 2000; Dunford JE, Thompson K et al., 2001] This inhibition has also been demonstrated for other nitrogen-containing bisphosphonates, namely zoledronic acid [Bergstrom JD, Bostedor

RG et al., 2000] and risedronate [Green JR, 2001; Thompson K, Coxon FP et al., 2001; Coxon FP, Dunford JE et al., 2001; Benford HL, Frith JC et al., 1999] and has also been demonstrated in vivo. [Frith JC, Armour KJ et al., 2001]

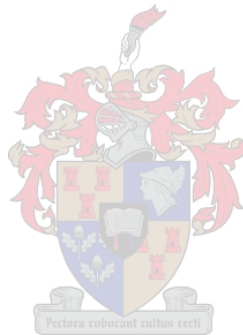
The effect of alendronate on osteoclasts can be prevented by the administration of geranylgeranylpyrophosphate and demonstrates that the inhibition of prenylation, and in particular the inhibition of geranylgeranylation, plays a major role in the inhibitory effect of alendronate on osteoclasts. [van Beek ER, Löwik C et al., 1999; Coxon FP, Helfrich MH et al., 2000; Fisher JE, Rogers MJ et al., 1999; Rogers MJ, Gordon S et al., 2000] Lovastatin and other statins can mimic the effects of alendronate on the osteoclast and this effect by statins can be reversed by the addition of geranylgeranylpyrophosphate but not farnesol - a clear indication that they prevent prenylation. [Fisher JE, Rogers MJ et al., 1999; Frith JC, Armour KJ et al., 2001; Luckman SP, Hughes DE et al., 1998; van Beek ER, Löwik C et al., 1999; Woo JT, Kasai S et al., 2000] Osteoclastogenesis can also be inhibited by alendronate and this effect can similarly be mimicked by statins. [Fisher JE, Halasy JM et al., 1998] There are therefore various lines of evidence demonstrating a similarity between the effect of alendronate and statins via their inhibition of prenylation. [Woo JT, Kasai S et al., 1998; Woo JT, Kasai S et al., 2000]

#### **4.2.2. Bisphosphonates and apoptosis**

The apoptosis of osteoclasts induced by bisphosphonates and statins, is associated with protein synthesis as well as the appearance of a caspase-3 protease-like activity. [Coxon FP, Benford HL et al., 1998; Benford HL, Frith JC et al., 1999; Benford HL, McGowan NW et al., 2001; Reszka AA, Halasy NJ et al., 1999] This rise in caspase-3 protease activity gives rise to cleavage and activation of a Mst-1 kinase that results in the formation of a 34-kDa species that is associated with the apoptosis of osteoclasts. [Laufs U and Liao JK, 1998; Lim L, Manser E et al., 1996; Reszka AA, Wesolowski G et al., 1998; Reszka AA, Halasy NJ et al., 1999] The activation of these kinases by the



aminobisphosphonates can be reversed by the addition of geranylgeraniol as well as farnesol. [Benford HL, Frith JC et al., 1998; Benford HL, Frith JC et al., 1999] However, these workers later show that the apoptosis of osteoclasts is prevented by inhibitors of geranylgeranylation but not by inhibitors of farnesylation, and they conclude that the apoptosis is mediated via a geranylgeranylation pathway. [Benford HL, McGowan NW et al., 2001] Conversely and surprisingly, other workers show that geranylgeraniol induces caspase-3-like activity. [Masuda Y, Nakaya M et al., 1997] Clearly the last word on the effect of prenylation inhibitors such as alendronate and statins on the osteoclast has not been written.



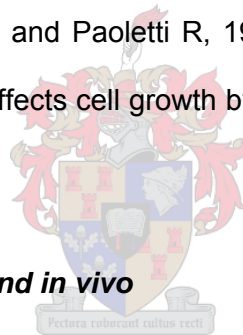
#### **4.2.3. Effects of statins on bone**

In the majority of the experiments demonstrating the role of prenylation in alendronate action, and investigating the effect of alendronate on bone, statins were used as controls. Osteoclast formation and activity was inhibited by both alendronate and lovastatin and this effect can be prevented by geranylgeranylpyrophosphate and mevalonate respectively. [Fisher JE, Rogers MJ et al., 1999] The ability of statins to mimic the effect of alendronate on bone, and that this occurs via the inhibition of prenylation, has been demonstrated by some authors. [Luckman SP, Hughes DE et al., 1998] Other researchers show that a statin, compactin, clearly affects the osteoclast by inhibiting the fusion of pre-osteoclasts and by inhibiting the formation of an actin ring. [Woo JT, Kasai S et al., 2000] They show that the dosage of compactin at which these effects are seen corresponds to the anti-resorptive dose of the compactin and that apoptosis of osteoclasts is not required for the anti-resorptive effect. They also show that the dose where these effects are seen is not the same as the dose where apoptosis of the osteoclast is observed. Consequently they show that the effects of compactin on the osteoclasts differs at different doses. Surprisingly they also show that these effects on osteoclast fusion and inhibition of the actin ring can be reversed by the addition of farnesol as well as geranylgeraniol whereas others show that certain effects of statins are only inhibited by geranylgeraniol. [Woo JT, Kasai S et al., 2000] The implication, therefore, is that different doses of a statin might have different effects on the osteoclast and that different pathways may be involved in these processes.

#### **4.2.4. Bisphosphonates and statins in metastases**

Osteoclasts play a major role in some cancer metastases where the osteoclasts are stimulated by the local production of the tumor peptide, parathyroid hormone-related peptide. [Mundy GR, 1997] The nitrogen-containing bisphosphonates, including

alendronate, are potent inhibitors of prenylation. [van Beek ER, Pieterman E et al., 1999] These agents are now frequently used as adjuvant therapy in oncology to inhibit metastases. [Body JJ, Bartl R et al., 1998] Their inhibition of prenylation and inhibitory effect on malignant metastases raises the possibility that they might have an effect on Ras, another prenylated protein. [Luckman SP, Hughes DE et al., 1998] However, although the aminobisphosphonates are potent inhibitors of osteoclast growth and differentiation, they mediate their effect on the osteoclasts by a mechanism other than via the inhibition of the oncogene Ras. [Coxon FP, Helfrich MH et al., 2000] Statins are also able to inhibit cell growth, have been investigated as adjuvants in cancer chemotherapy [Fisher JE, Rogers MJ et al., 1999; van Beek ER, Löwik C et al., 1999] and this anti-proliferative effect involves prenylation of proteins other than Ras. [Bellosta S, Ferri N et al., 2000b; Soma MR, Corsini A, and Paoletti R, 1992] This provides further evidence that the inhibition of prenylation affects cell growth by pathways other than those involving Ras.



#### **4.2.5. Effect of statins *in vitro* and *in vivo***

At the time of the conclusion of our studies, it was demonstrated, largely by the Mudy group, that bone formation is increased by simvastatin and that this occurs via an increased production of bone morphogenic protein-2 (BMP-2) by osteoblasts. [Mundy G, Gutierrez G et al., 1998; Mundy G, Garrett R et al., 1999] This led to a flurry of activity in this field. Unfortunately, this also led to numerous articles, not only in the lay press [1999b; 1999a] but also in the scientific literature, suggesting that statins will increase BMD and that statins may be used for the treatment of osteoporosis or for the prevention of osteoporosis. None of the results in the scientific literature could support these claims. Mundy and his co-workers did not make these claims. They did not at that time produce any data regarding the effect of statins on BMD in the rats that they studied. [Garret IR, Esparza J et al., 2000; Garret IR, Gutierrez G et al., 2001; Garret IR, Chen D et al., 2001;

Garrett IR, Gutierrez G, and Mundy GR, 2001; Mundy G, Gutierrez G et al., 1998; Mundy G, Garrett R et al., 1999; Mundy GR, 2001] Nonetheless, the work by Mundy *et al.* is a landmark study and focussed attention on the effect of statins on bone.

Further research done by Mundy and his co-workers confirmed that BMP-2 was involved in the effect of simvastatin on bone, and they also demonstrated that nitric oxide (NO) was involved in this process. [Garret IR, Esparza J et al., 2000; Garret IR, Chen D et al., 2001; Garrett IR, Gutierrez G, and Mundy GR, 2001; Whang K, Zhao M et al., 2000] The increase in BMP-2 caused by simvastatin has been confirmed by others [Sugiyama M, Kodama T et al., 2000] who also demonstrated that this could be produced by compactin but not pravastatin, indicating a possible differential effect of statins on bone.

The initial and subsequent work by Mundy et al. indicates that the effect of statins on the osteoblast is stimulatory. This occurs despite the inhibition of the small GTP binding protein Rho that is involved in cytoskeletal organisation. It would be expected that simvastatin may also have a stimulatory effect on other cells including osteoclasts but this effect not been demonstrated in other cells. Indeed the effect of statins on other cell lineages, including malignant cells, appears to be inhibitory. Statins have an inhibitory effect on various functions of macrophages. [Allen WE, Jones GE et al., 1997; Bellosta S, Ferri N et al., 2000a; Corsini A, 2000; Alfon J, Guasch JF et al., 1999] Statins also have an inhibitory effect on mesangial cell function and proliferation. [Ghosh PM, Mott GE et al., 1997; Ishikawa S, Kawasumi M, and Saito T, 1995; Kasiske BL, O'Donnell MP et al., 1994; O'Donnell MP, Kasiske BL et al., 1993] Mesangial cells and macrophages are polarised cells and belong to the same lineage as osteoclasts. Therefore, given the effect of statins on other cells of the same lineage, it would be expected that the inhibition of prenylation would have a similar inhibitory effect on osteoclasts. The question arises as to why the statins should stimulate the osteoblast and yet inhibit the osteoclast. In contrast to the above, we have clearly shown in more than one experiment that osteoclast function and

number is increased by simvastatin and also that the effect may be dosage dependent. To date these remain the only *in vivo* studies to specifically examine the effect of statins on different parameters of bone resorption.

Other workers have demonstrated that *in vitro*, compactin suppresses osteoclast function by inhibiting pre-osteoclast fusion. [Woo JT, Kasai S et al., 1998; Woo JT, Kasai S et al., 2000; Woo JT, Krecic AM et al., 2000] They demonstrate that this occurs via inhibition of actin ring formation and conclude that this is the result of lack of prenylation by the small GTP-binding proteins Rac and Rho.

#### **4.2.6. Effects on Rab proteins**

The above mechanisms all seem to involve prenylation of the small GTP-binding protein Rho. However, it is evident that other prenylated members of the Ras superfamily of small GTP-binding proteins, including Rab, may also be involved in bone turnover. Indeed it would be surprising if they were not involved in osteoclast function. Extensive intracellular vesicular trafficking is essential for the polarisation and bone resorbing activities of osteoclasts. It is therefore to be expected that the Rab proteins, intimately involved in vesicle targeting and trafficking [Kinsella BT and Maltese WA, 1991; Novick P and Zerial M, 1997] will play an important role in the function of these cells. [Väänänen HK, 2001; Abu-Amer Y, Teitelbaum SL et al., 1999] Rab-3 isoforms are expressed in bone marrow macrophages and their expression is enhanced by a variety of haemopoietic cytokines that promote the osteoclastic differentiation of these cells. [Abu-Amer Y, Teitelbaum SL et al., 1999; Väänänen HK, 2001] Of note is that the Rab-3 co-localises with the H<sup>+</sup>ATPase or the vacuolar proton pump of osteoclasts. [Abu-Amer Y, Teitelbaum SL et al., 1999] The involvement of other Rab proteins in osteoclast function has been demonstrated. Anti-sense nucleotides against Rab-7 have resulted in a reduction in the number of resorbing osteoclasts, and significantly inhibited osteoclastic bone resorption *in vitro*. [Zhao HB, Ettala O, and Väänänen HK, 2001]

The nitrogen-containing bisphosphonates, including risedronate, have been demonstrated to inhibit farnesylpyrophosphate synthase and to potently inhibit prenylation. [van Beek ER, Pieterman E et al., 1999] As such they can also be expected to inhibit prenylation of Rab. An analogue of risedronate has been shown to be an inhibitor of GGTase II, a prenyl transferase that is intimately involved in the prenylation of Rab, and which is the only bisphosphonate tested that has this activity. [Coxon FP, Dunford JE et al., 2001; Coxon FP, Helfrich MH et al., 2001] Although this bisphosphonate also weakly inhibits farnesylpyrophosphate synthase, it results in a selective loss of geranylgeranylation of Rab proteins. The inhibition of Rab prenylation and the inhibition of Rab function by statins has previously been demonstrated. [Kinsella BT and Maltese WA, 1992] It is clear that Rab proteins play an important role in osteoclast function. Their inhibition might be an important method by which certain drugs, including statins, exert their effect on bone.

The above data illustrate the similarities between the modes of action between the aminobisphosphonate and the statins. The data also indicate that, although the effect of statins on osteoblasts is stimulatory, there is sufficient evidence to suggest that the effect of statins on osteoclasts should be inhibitory. However, there are many variables that may influence the effect of statins on bone and result in effects other than those indicated above. So it may be that under certain circumstances a statin may cause inhibition of the osteoblast and stimulation of the osteoclast. These variables include the type of statin used, the dosage of the statin used, the differential effects of nitric oxide on osteoblasts and osteoclasts, and the effects of statins on BMP-2. These additional signalling molecules and signalling pathways have received increasing attention in the control of bone metabolism. They have also been implicated in the mechanisms by which prenylation inhibitors and statins act on bone. These factors will be discussed in later paragraphs.

### **4.3. The demonstrated effect of statins on bone.**

#### **4.3.1. Effect of statins on bone formation**

We have shown that simvastatin 20mg/Kg/day significantly affects parameters of bone turnover in non-ovariectomised rats. This was demonstrated in the sham-operated group (Sh-S) in the ovariectomy model (Fig. 3.1.2) and was reproduced in a further study using the same dose in intact rats (S20) (Fig. 3.2.2). QBH parameters of bone formation were increased and this is especially evident when looking at the percent change over the controls induced by simvastatin (Figs. 3.1.4 and 3.2.4).

This increase in bone formation is supported by the work of Mundy and his co-workers. [Mundy G, Gutierrez G et al., 1998; Mundy G, Garrett R et al., 1999] Their treatment with similar doses of simvastatin result in a 39% increase in trabecular bone volume in intact rats and a 25% to 94% dosage-dependant increase in trabecular bone volume in ovariectomised rats. An increase in new bone formation is induced by statins when added to neonatal murine calvarial bones in organ culture, as well as when injected into the subcutaneous tissue overlying murine calvaria. Furthermore, the effect of systemic administration of statins was investigated in ovariectomised and intact rats as measured by histomorphometric parameters. Simvastatin in dosages ranging from 1mg/Kg/day to 10mg/Kg/day were reported, and these doses increase bone formation rate and trabecular bone volume. They also clearly demonstrate that simvastatin is able to activate the promoter region of the bone morphogenetic protein-2 (BMP-2) gene and increase the expression of BMP-2 mRNA in a specific fashion. [Mundy G, Garrett R et al., 1999] This effect on BMP-s is also demonstrated for mevastatin and fluvastatin. Of note is that they do not see any differences in the effects of the different statins.

Mundy *et al.* state that they investigated the effect of simvastatin with doses up to 50mg/Kg/day. [Mundy G, Garrett R et al., 1999] Unfortunately the effect of these large

doses on bone are not reported. Also, the effect of simvastatin on the bone formation rate of their intact rats is also not reported.

#### **4.3.2. Effect of statins on bone resorption**

In addition to increasing bone formation, we have also shown an increase in parameters of bone resorption with many doses of simvastatin, and for more than one statin. This was demonstrated with the sham-operated group of the ovariectomy model (Sh-S) (Fig 3.1.3) and again reproduced in the intact rat group (S20) (Fig 3.2.3; Figs. 3.1.4 and 3.2.4). This is in contrast to the work of Mundy et al. who found that osteoclast numbers were reduced. [Mundy G, Gutierrez G et al., 1998; Mundy G, Garrett R et al., 1999] Unfortunately they seem to indicate in their article that osteoclasts and their function were not completely assessed in their experiments. Where these figures are reported, they show that osteoclast numbers are decreased in the intact as well as the ovariectomised rats. As will be seen later, this is an important observation. They also comment that the effect on the osteoclasts seems to be minor in comparison with the effect on bone formation. [Mundy G, Garrett R et al., 1999] The above findings of increased bone formation and resorption in our experiments apply only to simvastatin at a dose of 20mg/Kg/day. As will be shown later, at lesser doses different effects are observed on these parameters of bone metabolism.



#### **4.3.3. Effect of statins on BMD**

We have also shown that three classes of statins, two lipophilic and one hydrophilic, decrease BMD. Our findings that statins decrease BMD are supported by our data that statins significantly increase bone resorption whereas effects on bone formation are modest.

Very little *in vivo* research on statins and bone has been done in laboratory animals. One published study supports the hypothesis that statins will increase BMD. They looked at compressive strength of the vertebrae in rats and found that the compressive strength was increased in those rats treated with oral simvastatin 10mg/Kg/day over 3 months. [Oxlund H and Andreasse TT, 2000] A further study in laboratory animals indicates that a statin was not able to prevent the bone loss induced by ovariectomy in laboratory rats [Yao W, Li CY et al., 2001] These researchers used doses of simvastatin ranging from 0.3mg/Kg/day to 10mg/Kg/day in ovariectomised rats and the results show there were no changes in the bone volume, bone formation rate and eroded surfaces. This supports our findings that simvastatin was not able to prevent the bone loss which occurs after ovariectomy and also supports our supposition that the effect of simvastatin on bone may require the permissive effect of oestrogens. Other researchers, however, using the same dose of simvastatin, 10mg/Kg/day, were able to demonstrate that simvastatin was able to prevent the bone loss induced by ovariectomy. [Jiang Y, Zhao Y et al., 2001] Similarly, another group of researchers were able to demonstrate that simvastatin in similar and equivalent doses was able to partially prevent the bone loss after ovariectomy. [Jiang Y, Zhao Y et al., 2001; Masarachia PJ, Wesolowski G et al., 2001] Furthermore, they also demonstrate that atorvastatin 10mg/Kg/day was not able to prevent this bone loss in the same manner as simvastatin and suggests a possible differential effect of statins on bone similar to our findings.

In summary, it is evident from available data that statins affect bone, but that the effect seems to vary. What appears to be constant is that statins increase bone formation, whereas effects on bone resorption vary. These differences may be explained by differences in experimental design and certainly require more research. [Oxlund H, Dalstra M, and Andreassen TT, 2001]

#### **4.4. The effect of different doses of simvastatin on QBH parameters.**

We have shown, for the first time, that the effect of simvastatin on parameters of bone formation and bone resorption differs according to the dose of simvastatin administered.

##### **4.4.1. Different doses examined.**

Mundy *et al.* administered simvastatin in doses ranging from 1mg/Kg/day to 50mg/Kg/day in their *in vivo* experiments. [Mundy G, Garrett R et al., 1999] Unfortunately, not all the results pertaining to the different doses are reported. In particular, no indication is given regarding the effect of the very large doses of simvastatin on the parameters of bone turnover. It would appear that the different doses of simvastatin were administered in different experiments and therefore no direct comparisons can be made between the effects of the different doses of simvastatin on the different parameters of bone turnover. No comparison is consequently available between the effect of the smallest simvastatin dose, 1mg/Kg/day, and simvastatin 50mg/Kg /day. Nonetheless, from the data there seems to be a big difference in the increase of bone volume between simvastatin 1mg/Kg/day and 10mg/Kg/day, the former being less than the latter. No comparable data exist for different different dosages of compactin, simvastatin and pravastatin, or any other statins. [Sugiyama M, Kodama T et al., 2000; Woo JT, Kasai S et al., 2000]

We have shown that with simvastatin 20mg/Kg/day (Sh-S, S20), bone formation is increased compared to the matching controls (Fig. 3.3.4; Table 3.3.1). Thereafter, with decreasing doses of simvastatin bone formation also decreases. With 10mg/Kg/day bone formation is suppressed, and is already less than the amount seen in the control group. This suppression of bone formation is further evident with smaller doses of simvastatin, 5mg/Kg/day and 1mg/Kg/day. Our data also demonstrate that simvastatin increases bone resorption at a dose of 20mg/Kg/day (Fig. 3.3.6; Table 3.3.1). Thereafter bone resorption steadily decreases with decreasing doses of simvastatin. At 5mg/Kg/day bone resorption is clearly suppressed and is less than that seen in the control group. However, with simvastatin 1mg/Kg/day there is again an increase in parameters of bone resorption above the levels seen in the control group.

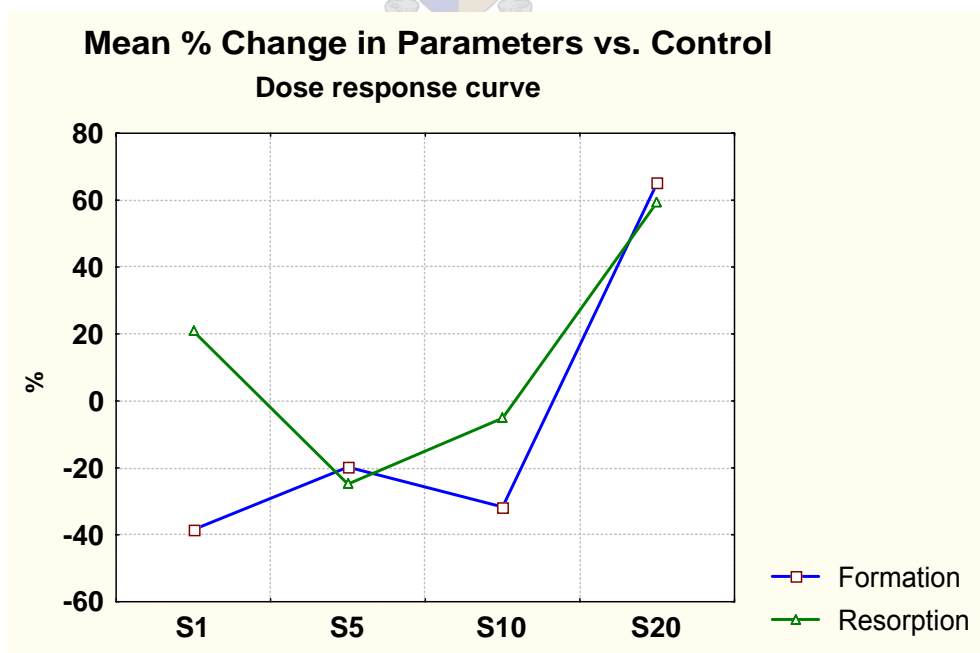
#### **4.4.2. Biphasic response**

It is clear from these results that there is a biphasic effect of simvastatin on both resorption and formation; at one dose there is an increase of bone formation and at another there is an inhibition of bone formation (and not merely a lesser increase of bone formation). A similar biphasic phenomenon is evident for the parameters of bone resorption. This is an important observation. There is no immediate precedent for this effect and it needs to be explained. Plausible explanations are offered in a later part of this chapter.

It is true that the doses of simvastatin used in our studies do not go low enough, and do not follow back to the dose of simvastatin where there is no effect on the particular parameter of bone turnover. At the time that these studies were started, we had no idea what the minimum effective doses of simvastatin would be. Nonetheless, a dose response curve for the existing doses could be constructed (Study 3.3; Figs. 3.3.3., 3.3.6., 4.4.).

#### **4.4.3. Dose-response curves**

Another way of looking at the data is to view the percent change over the control value of any parameter, or stated another way, the delta value expressed as a percent of the control. Considering the QBH parameters of formation, they all change in the same direction and in parallel for all the different doses of simvastatin (Fig 3.3.4). In addition, the percent changes for all the parameters of bone formation are nearly of the same magnitude - no parameter has a percent change that is dramatically more than the percent change of any other parameter for any particular dose. Therefore the mean percent change of all the formative parameters for any particular dose of simvastatin relatively accurately describes the percent change seen with any individual formative parameter at that particular dose. The same can be said for the QBH parameters of bone resorption (Fig. 3.3.6). From these mean formative and resorptive values, a dose response curve for bone formation and for bone resorption can be constructed (Fig. 4.1.). It is evident that the dose-response curves for the QBH parameters of bone formation are not the same as the dose-response curves for bone resorption (Fig. 4.1.). As will be seen, this may have important implications for the BMD measured in these animals.



**Figure 4.1.** Dose response curve for the QBH parameters of formation and resorption.

These findings that different doses of statins have different effects on the parameters of bone formation and resorption will have important consequences for the interpretation of existing data. They will also have an important impact on the planning of future studies that aim to look at the effect of statins on bone health.

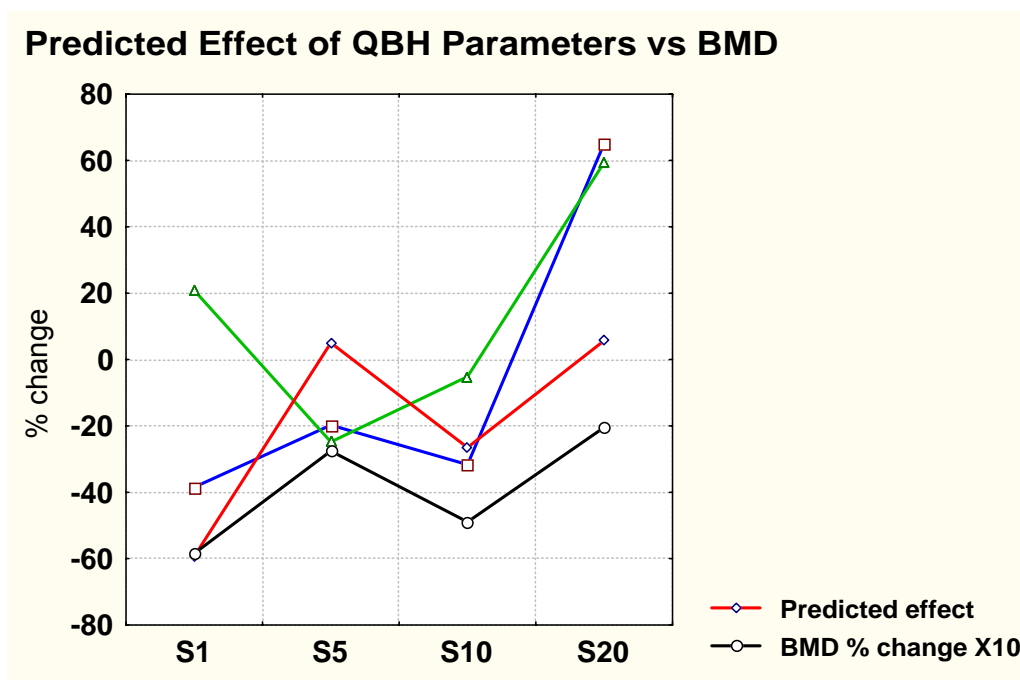
#### **4.5. The effect of different dosages of simvastatin on BMD**

We have clearly been able to show that the effect that simvastatin has on BMD differs with different doses of the drug. This is the first set of data to show this effect and is also the only study to have looked at this effect.

In our studies the influence of simvastatin on the BMD in the non-ovariectomised rats differs with different doses of simvastatin. At a dose of 20mg/Kg/day simvastatin, there was a downward trend in the BMD in the groups treated with simvastatin (Sh-S, S20) compared to their controls (Sh, C) but this was not statistically significant. This suggests that simvastatin caused a balanced increase in both formation and resorption at this dose. Thereafter there was a progressive decrease in BMD with decreasing doses of simvastatin. At a dose of 1mg/Kg/day, simvastatin produced a significant reduction in BMD. This means that the largest reduction in BMD occurs with the smallest dose of simvastatin.

When the changes in QBH parameters of formation and resorption that are induced by simvastatin 1mg/Kg/day, are considered together, then the resultant BMD induced by simvastatin 1mg/Kg/day can be qualitatively predicted; resorption is increased and formation is reduced, resulting in a reduction of BMD. The BMD measured at any particular dose of simvastatin can also be expressed as a percent change of BMD from control - put another way, the delta value expressed a percent of the control value. In this way a dose-response curve for BMD is described.

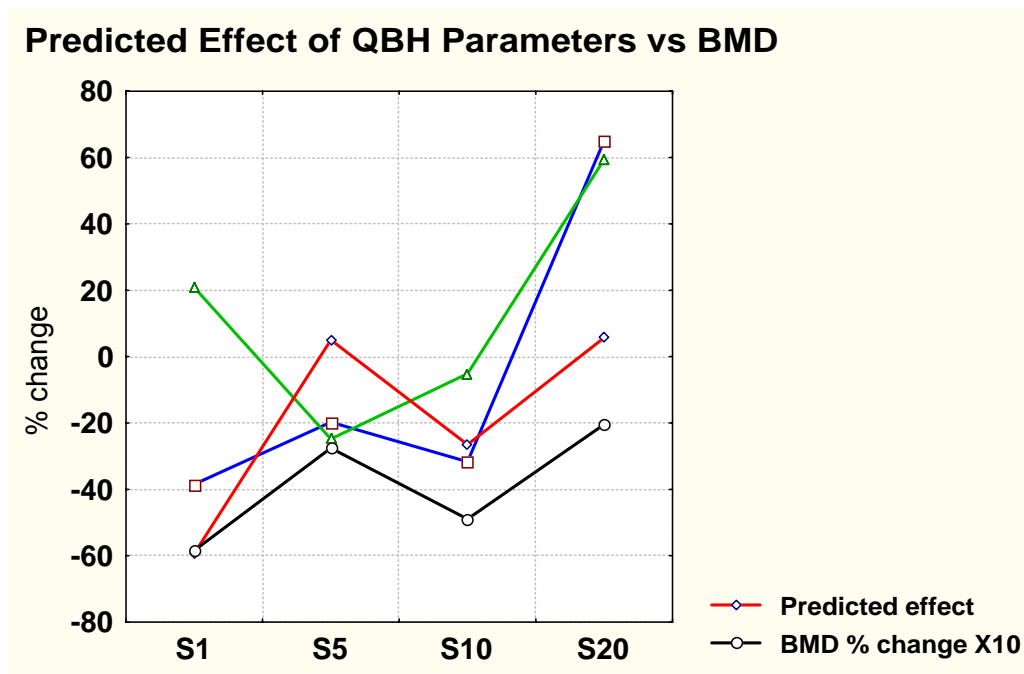
It should be possible to predict the change in BMD from the summated influences of bone formation and bone resorption e.g. if formation and resorption were to be increased, but resorption were to outstrip formation, then the BMD would be predicted to decrease. Such a supposed predicted-effect curve can be constructed by summing the previously described dose percent-response curves for formation and resorption (Fig. 4.3). This curve should then reflect the changes seen in BMD, at least qualitatively if not quantitatively. Indeed, if this predicted change in BMD is compared to the actual percent change in the BMD (multiplied by 10 for graphical purposes) then there is a remarkable similarity between the two curves (Fig. 4.4.). The curves parallel each other and the predicted curve accurately reflects what did actually happen to the BMD.



**Figure 4.2. Predicted change in BMD deduced from the summation of the formation and resorption dose %-response curves.**

The above method to describe the predicted BMD from the summation of the changes in the QBH parameters of formation and resorption has not been used before. Whether this is a valid method to describe the predicted change in BMD is debatable. It also assumes that the effects on resorption and formation, which we assesses at weeks 8

and 12, remain unchanged throughout the entire study period. A 100% increase in the parameters of formation does not necessarily offset a 100% increase in resorption parameters and consequently lead to no change in the BMD. In this sense it cannot be used to quantitatively predict the resultant change in BMD. However, qualitatively it does give an indication regarding what should be happening to the BMD and from that point of view gives some kind of rough idea as to the accuracy of the findings. It is evident that in our studies, if not validly, then certainly serendipitously, this method did accurately predict the change in BMD that did occur.



**Figure 4.3.** The predicted change in BMD compared to the actual % change in BMD.

#### 4.6. Studies in humans

At the time of the first announcement of their findings by Mundy et al. [Mundy G, Gutierrez G et al., 1998] some data was presented outlining a meta-analysis of a large number of patients in two ongoing prospective studies, the Study of Osteoporotic

Fractures (SOF) and the Fracture Intervention Trial (FIT). [Bauer DC, Mundy G et al., 1999] The authors concluded that statin use was possibly associated with a higher BMD and a reduced fracture risk. They were able to show this for the more potent lipophilic statins, but not for pravastatin, and that this effect was not seen with non-statin lipid-lowering drugs. Shortly thereafter, there was a flurry of reports on the same topic and to date there have been 23 studies which explored the hypothesis that statins will improve bone health.





#### **4.6.1. Case controlled observational studies with fracture risk as endpoint**

Three studies were case-controlled observational studies which looked at the fracture risk as an endpoint in a large number of patients from UK-based General Practice Research Database (GPRD) [Meier CR, Schlienger RG et al., 2000], the Medicare and Medicaid Pharmacy assistance program [Wang PS, Solomon DH et al., 2000] and six health maintenance programs in the USA. [Chan KA, Andrade SE et al., 2000] They all concluded that the use of statins was associated with a reduced fracture risk. Furthermore they were able to show that this reduced fracture risk was not conferred by the use of non-statin lipid-lowering agents.

Four further similar studies were unable to confirm an association between statin use and fractures. These studies looked at large numbers of patients from the Women's Health Initiative Observational Study, [LaCroix AZ, Cauley JA et al., 2000] the GPRD database ( the same database as studied by Meier et al.) [van Staa TP, Wegman SLJ et al., 2000; van Staa TP, Wegman S et al., 2001b] and two large statin and cardiovascular studies, the LIPID Study [Reid IR and Haugue W, 2000; Reid IR, Haue W et al., 2001] and the 4S study. [Pedersen TR and Kjekshus J, 2000] These researchers conclude that statin use did not change the fracture risk in these patients.

The results of these trials have been the source of much debate. It appeared to be strange that two studies looking at the same GPRD database found such diametrically opposite results [Meier CR, Schlienger RG et al., 2000; van Staa TP, Wegman S et al., 2001b] which remained even after re-analysis of different permutations of the data. [van Staa TP, Wegman S et al., 2001a] These studies were subjected to further critique. [Cummings SR and Bauer DC, 2000; Hennessy S and Strom BL, 2001; Mundy GR, 2001] It is well known that an increased body mass is protective against fractures [Barengolts EI, Karanouh D et al., 2001] and that those subjects with the most unfavorable BMD

have a lower weight and a more favourable lipid profile, whereas those who are overweight tend to have dyslipidaemia, and consequently take statins, have the most favourable BMD. [Adami S, Braga V et al., 2001b; Adami S, Braga V, and Gatti D, 2001a] [Solomon DH, Finkelstein JS et al., 2001; Cauley JA, Jackson R et al., 2000] Even allowing for the possible confounding effects of body mass index (BMI) in reanalyses, this made no difference to the conclusions drawn. [Hennessy S and Strom BL, 2001] The study by Meier et al. [Meier CR, Schlienger RG et al., 2000] found that short-term statin use, even after only 1 month, also conferred a reduced fracture risk and it is inconceivable how an anabolic agent like a statin could strengthen bones to this degree over such a short time. Nonetheless, a different meta-analysis was done on the 8 trials completed by this time and they concluded that the use of statins did confer protection against fractures. [Bauer DC, Black DM, and van der Klift M, 2001] There has been some anecdotal evidence, that pravastatin does not affect bone metabolism and that many of the effects seen on bone with other statins are not seen with pravastatin. [Cauley JA, Jackson R et al., 2000; Watanabe S, Fukumoto S, and Takeuchi Y, 2000](Mundy G - personal communication, April 2001). If this is indeed so then it is not surprising that no protection against fractures was seen in the Lipid Trial as this cardiovascular trial utilised pravastatin. [Reid IR and Haugue W, 2000; Reid IR, Haue W et al., 2001]

#### **4.6.2. Studies with BMD as endpoint**

Although the above 8 trials all looked at fractures as the end-point, a further important study analysed both the fracture risk and measured the BMD in a large cohort from the Barwon Statistical Division in Australia. [Pascoe JA, Kotowicz MA et al., 2001] These authors find that the use of statins is associated with a small increase in BMD of the femoral neck only, and a decreased risk (odds ratio of 0.42) of sustaining a fracture. However, they conclude that this large decrease in fracture risk in statin users cannot be

explained by the modest increase in the femoral BMD. They advise that other reasons be sought for the reduction in the fracture risk.

There are many reasons, other than an increase in BMD, why statin use could be associated with a reduced fracture risk. It is very likely that statins are prescribed to patients who are more likely to have a longer survival. Those patients who, for whatever reason or underlying illness, are unlikely to have a prolonged survival are less likely to receive a statin. These patients, due to their associated co-morbidities, are also more likely to be osteopaenic. There is therefore a bias for patients with healthier bones to receive statins.

Fractures are related to falls, and falls in the elderly are frequently caused by transient cardiac dysrhythmias precipitated by ischaemia or heart disease. Statins reduce these cardiac dysrhythmias, improve transient ischaemia [van Boven AJ, Jukema JW et al., 1996] and have been shown have to have a favourable affect on the QT dispersion, which plays a role in these arrhythmias. [Mark L and Katona A, 2000] Consequently, statin-users are less likely to fall from cardiac causes. Statins also have neuroprotective effects and may improve cognition in elderly patients. [Vaughan CJ and Delanty N, 1999] The confounding effect of BMD has already been alluded to. There is also a likelihood that people who take statins regularly also start taking medications and other measures which reduce the fracture risk.

The first study to look at the effect of statins on BMD rather than fracture risk, investigated the effect of statins on the BMD in type 2 diabetics. [Chung YS, Lee MD et al., 2000] They were able to conclude that statin use was associated with a higher BMD. Seven further studies investigated the effect of statins on BMD. Four of these studies were able to show that statin use was associated with an increase in BMD compared to controls. [Barengolts EI, Karanouh D et al., 2001; Cauley JA, Jackson R et al., 2000; Edwards CJ, Hart DJ, and Spector TD, 2000; Watanabe S, Fukomoto S, and Takeuchi Y,

2000] Of these, two showed that the use of pravastatin was not associated with any increase in the BMD. [Cauley JA, Jackson R et al., 2000; Watanabe S, Fukumoto S, and Takeuchi Y, 2000] One of these studies measured biochemical markers of bone turnover in addition to the BMD, in a group of patients > 55 years of age. [Watanabe S, Fukumoto S, and Takeuchi Y, 2000] These researchers show that 3 months of treatment with fluvastatin significantly decreased urinary N-terminal telopeptide (NTx) whereas pravastatin did not have an effect. Neither bone-specific alkaline phosphatase nor osteocalcin were affected by the statins used. Only the BMD of the lumbar spine, not the rest of the skeleton, was increased with fluvastatin, whereas BMD of the lumbar spine decreased with pravastatin. Furthermore they show that the effect of the statins is predominately seen in females. This is intriguing in that the effect was seen only in patients in whom estrogen was absent. The remaining 3 studies [Sirola J, Honkanen R et al., 2001; Solomon DH, Finkelstein JS et al., 2001; Yaturu S, Alferos MG et al., 2001] investigated the effect of statins on BMD in large groups of patients, including a cohort from the Kuopio Osteoporosis Risk Factor and Prevention Study in Finland [Sirola J, Honkanen R et al., 2001]. None of these studies were able to find any association between statin use and an increase in BMD.

#### **4.6.3. Studies investigating the effect on biochemical markers of bone turnover**

Six other studies investigated the effect of statins on biochemical markers of bone turnover. Salbach et al. found that atorvastatin decreased bone-type alkaline phosphatase in the first 3 days, which then returned to baseline by day 30. No effect was seen on osteocalcin or urinary carboxyterminal telopeptide (CTx). They also found that the effect was most pronounced in males - in contrast to a previous study. [Salbach P, Kreuzer J, and Seibel MJH, 2001] Rejnmark et al. show that statin use is associated with 16% higher parathyroid hormone levels and that all the biochemical markers of bone turnover, namely osteocalcin, bone-type alkaline phosphatase and CTx, were decreased in statin

users. They conclude that statins reduce bone turnover. [Rejnmark L, Buus NH et al., 2001] Fluvastatin was investigated in another trial and was found not to influence biochemical markers of bone turnover. [Bjarnason NH, Shalmi M et al., 2000; Bjarnason NH, Riis BJ, and Christiansen C, 2001] Whereas the patients included in the above 3 trials were relatively small, Stein et al. measured the stored serum samples from a large cohort of patients who were included in a trial comparing the safety and lipid-lowering effect of 40mg to 80mg of simvastatin with 20mg to 40mg of atorvastatin. [Stein EA, Farnier M et al., 2001] They found that simvastatin, but not atorvastatin, significantly decreased the bone-specific alkaline phosphatase in both males and females by 4% - 7% and that this appeared to be a dose-dependent effect with a greater reduction on simvastatin 80mg. Simvastatin caused a non-significant reduction in urinary CTx also with an apparent dosage effect. Atorvastatin had no effect on these biochemical markers. The authors remark that these effects are beneficial in those using simvastatin. However, these effects could equally be detrimental. Paradoxically, EA Stein has also stated that investigations into large databases of lipid-lowering trials involving simvastatin have shown no effect of simvastatin on alkaline phosphatase. (Personal communication, Sept 1988, Cardiology Congress, Durban.)

In contrast, a further prospective study on a small group of patients showed a significant increase in osteocalcin levels in those patients using simvastatin 20mg per day. No effect was seen on bone alkaline phosphatase, urine deoxypyridinoline and urinary NTx. [Chan MH, Mak TW et al., 2001] Another well designed prospective randomised trial investigated the effect of cerivastatin 0.4mg per day on biochemical markers of bone turnover in patients over 12 weeks. [Cosman F, Nieves J et al., 2001] These researchers measured osteocalcin, propeptide Type I procollagen, NTx and CTx and found no significant change in any parameter.

#### **4.6.4. In summary.**

Overall, the studies in humans on fracture rate, BMD and biochemical markers of bone turnover show no consistent effect. They have no uniform design and many can be criticised for their poor execution. Furthermore, none of these studies confirm or refute, any of the data obtained in laboratory animals. This could mean that statins have differing effects on bone which differ according to the circumstances under which the study was performed, with variables, such as dose of the statin, the kind of statin, the bioavailability of the statin and concomitant medication all playing a role. We have already shown that the dose of the statin plays a role in the effect of statins on bone. Presumably this reflects the amount that reaches the bone/plasma interface and therefore means that bioavailability must also play a role. What is required is a large prospective trial, which is thoughtfully designed, specifically to explore the influence of statins on bone in humans.

#### **4.7. Mechanisms by which statins could affect bone**

We have clearly demonstrated that statins affect bone and mineral metabolism albeit in a complex manner. This supports the work of others who have shown that simvastatin increases bone formation. [Mundy G, Gutierrez G et al., 1998; Mundy G, Garrett R et al., 1999] Statins other than simvastatin also affect bone. [Cosman F, Nieves J et al., 2001; Gasser JA, 2001; Miller SC, Bowman BM, and Bagi C, 2001; Sato.M., Schmidt A et al., 2001] The question arises, "By what mechanism do statins affect bone and mineral metabolism?".

It would be wrong to group all the statins into this answer as there is reason to believe that some statins might behave in a different fashion from simvastatin. Our data and that of others pertain mostly to simvastatin. It is also quite possible that the biomolecular effect of statins on osteoblastic bone formation may differ from those seen

with osteoclastic bone resorption. Statins have been shown to have different effects on different cell lines. [Newman A, Clutterbuck RD et al., 1994]

Although we stated in the preamble to our hypotheses that the effect of statins on bone would involve the inhibition of prenylation, we certainly have not proven this in our studies. The effect of statins on bone could have been as a result of the inhibition of HMG-CoA reductase, thereby reducing the downstream components of the cholesterol synthetic pathway. Alternatively the effect of simvastatin on bone could have been due to a mechanism that has nothing to do with its inhibition of HMG-CoA reductase. It could have been due to another effect that has not become apparent, or it could even have been due to a toxic effect of the drug.

There are no reports in the literature indicating a consistent toxic effect of simvastatin on rats at the doses used in our studies. There is also no reported data to indicate that the effects of statins on bone may be due to a toxic effect. Although no other organ examinations were done in our rats, and apart from one unexplained death in one rat, our rats appeared to be healthy. A strong argument against a toxic effect is that the largest effect on BMD were seen with the smallest doses of simvastatin.

Many receptors and proteins involved in signal transduction pathways are prenylated and are therefore targets for prenylation inhibitors including statins. Simvastatin has been demonstrated to inhibit sterol synthesis and attenuate progesterone secretion by human granulosa cells. [van Vliet AK, van Thiel GC et al., 1996] Given that the effect of simvastatin on bone in our experiments were the largest in non-ovariectomised rats, this raises the possibility that simvastatin may have caused hypogonadism. However, the oestrogen and rFSH levels of Study 3.1 clearly indicate that this is not the case (Table 3.1.2.).

#### 4.7.1. Multiplicity of effects

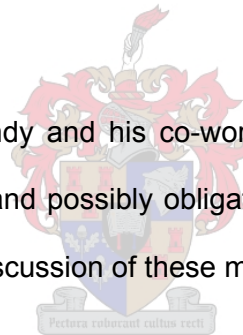
Mundy and his co-workers confirm that simvastatin increases bone formation and demonstrated that this is due to an increase in BMP-2 production induced by the simvastatin. [Mundy G, Gutierrez G et al., 1998; Mundy G, Garrett R et al., 1999; Garret IR, Esparza J et al., 2000; Garret IR, Chen D et al., 2001; Gutierrez G, Garret IR et al., 2001; Whang K, Zhao M et al., 2000; Garrett IR, Gutierrez G, and Mundy GR, 2001] They have demonstrated that it is the active form of simvastatin and not the inactive prodrug which stimulates bone formation, indicating that the inhibition of HMG-CoA is paramount in the process of stimulation of bone formation by simvastatin. [Garret IR, Esparza J et al., 2000; Garret IR, Chen D et al., 2001] This was further confirmed by their finding that the process was inhibited by the addition of mevalonate or geranylgeranylpyrophosphate to their cell cultures. Because Rho is geranylgeranylated, it was supposed that Rho was involved and they could show that the process was blocked by the addition of the specific Rho inhibitor, *Clostridium botulinum* C3 transferase. They demonstrated that the bone formation was blocked by NOS inhibitors, indicating that NO signalling was important and also concluded that the eNOS activation was the result of Rho inhibition. The NO in turn leads to increased BMP-2 expression. In addition they convincingly showed that BMP-2 was essential in the process as the process did not take place in the presence of cells with inactive BMP-2 receptors. Lastly, BMP-2 leads to osteoblast differentiation and bone formation. [Garret IR, Esparza J et al., 2000; Garret IR, Chen D et al., 2001]

Since their initial publication, Mundy and his co-workers have also clearly confirmed that the inhibition of prenylation is involved in this process of stimulation of bone formation by simvastatin. [Garret IR, Esparza J et al., 2000] This supports our initial hypothesis that simvastatin would affect bone metabolism via an inhibition of prenylation. However, the end result of the inhibited prenylation demonstrated by these researchers is



different from what we had hypothesised. We proposed that the inhibition of prenylation would inhibit Rho function in these bone cells. This has clearly been confirmed by Mundy et al. [Garret IR, Esparza J et al., 2000] Based on previous research by others we further proposed that this inhibition of Rho function would lead to an inhibition of cell growth. [Burrige K and Chrzanowska WM, 1996; Hotchin NA and Hall A, 1996; Laufs U, Marra D et al., 1999; Lebowitz PF, Casey PJ et al., 1997; Olson MF, Ashworth A, and Hall A, 1995; Symons M, 1996] What emanates from the work of Mundy et al. is that Rho inhibition causes, or is associated with, other effects such as an increase in NO production which is able to override the suppressive effect of Rho inhibition on cell growth and lead to stimulation of growth. If the effect on the osteoclast is similar, then it is quite conceivable that simvastatin should stimulate osteoclastic bone resorption. This we have indeed demonstrated.

From the studies by Mundy and his co-workers it is also clear that BMP-2 and nitric oxide (NO) are intimately, and possibly obligatorily, involved in mediating the effect of simvastatin on bone. A brief discussion of these mechanisms is warranted.



#### **4.7.2. Bone Morphogenetic Proteins**

Bone Morphogenetic Proteins (BMP) are cytoplasmic proteins found in chondrocytes, osteoblasts and osteoclasts. BMP-2 provides a tonic baseline control of the rate of bone remodeling by promoting osteoblast differentiation and also osteoblastogenesis. [Abe E, Yamamoto M et al., 2000] This stimulatory effect of BMP-2 on osteoblasts has been well established. However in addition, BMP-2 also increases osteoclastogenesis and activates osteoclasts, possibly with the assistance of stromal cells. [Kanatani M, Sugimoto T et al., 1995]

Simvastatin stimulates osteoblast numbers and bone formation via an increase in BMP-2 expression [Mundy G, Gutierrez G et al., 1998; Mundy G, Garrett R et al., 1999;

Garret IR, Chen D et al., 2001] and this has been confirmed by other researchers. [Sugiyama M, Kodama T et al., 2000] It has further been demonstrated that the effect of the statin on bone is mediated via inhibition of HMG-C0A reductase and also by the resultant inhibition of Rho. This is associated with an increase in eNOS expression, which in turn results in an increase in BMP-2 transcription. [Garret IR, Esparza J et al., 2000] These effects have been demonstrated, not only for simvastatin, but also for other statins such as compactin [Sugiyama M, Kodama T et al., 2000] and lovastatin. [Garret IR, Esparza J et al., 2000] However, these effects could not be demonstrated for hydrophilic pravastatin *in vitro*. [Sugiyama M, Kodama T et al., 2000] This indicates that there might be a differential effect by statins on bone, possibly dictated by bioavailability and other properties of these drugs.

Apart from increasing the expression of BMP-2, statins are able to induce other bone genes such as osteocalcin, alkaline phosphatase and osteopontin. [Carley W and Phan S, 2001] It also appears as if different statins have different effects on the activation of the different bone genes; messenger RNA for osteocalcin, alkaline phosphatase, osteopontin and BMP-2 were increased by cerivastatin whereas only alkaline phosphatase and BMP-2 were increased by atorvastatin, and only BMP-2 and osteocalcin were increased by simvastatin. [Carley W and Phan S, 2001] This suggests that there may be promoter thresholds that differ between statins. Mundy and his co-workers found that simvastatin increased the expression of BMP-2 but not of BMP-4, interleukin-6 or of the parathyroid hormone (PTH)-related peptide, and they were of the opinion that the effects of statins were rather specific for the BMP-2 gene. [Mundy G, Garrett R et al., 1999] Nonetheless, the question still arises whether the effect of statins on bone may not also, at least partially, be due to an effect on the promoter region of bone genes other than BMP-2.

It is clear therefore that statins have a profound effect on bone metabolism and that there seems to be a differential effect produced by the different statins. These differential effects may be the result of different effects of statins on signalling molecules such as BMP-2 and other bone gene products, or of differing chemical composition and half life resulting in differing concentrations reaching the bones.

#### **4.7.3. Nitric oxide signalling and the influence of caveolae.**

Nitric oxide (NO) is involved in several distinct signalling pathways in blood vessels:-

- Endothelium-dependent vasodilation
- Cytokine/endotoxin-induced vasodilation
- Nerve-dependent vasodilation

Nitric oxide is produced from arginine by a specific homodimeric enzyme, nitric oxide synthase (NOS)(Fig. 4.2.). [Knowles RG and Moncada S, 1992] The above three processes that lead to vasodilation are partially the result of three distinct isoforms of NOS, which differ in the way that they are stimulated. Two of these NOS isoforms, those involved with endothelium-dependent (eNOS) and nerve-dependent vasodilation (nNOS), are constitutive and the NOS involved with cytokine-dependent vasodilation is inducible (iNOS). The NOS reaction produces NO from L-Arginine in a complex reaction which incorporates O<sub>2</sub> into NO and citrulline, and utilises NADPH, FMN, FAD, tetrahydrobiopterin, non-haem iron. For the endothelial form of NOS, Ca<sup>++</sup> and calmodulin are also required and essential. [Knowles RG and Moncada S, 1992]

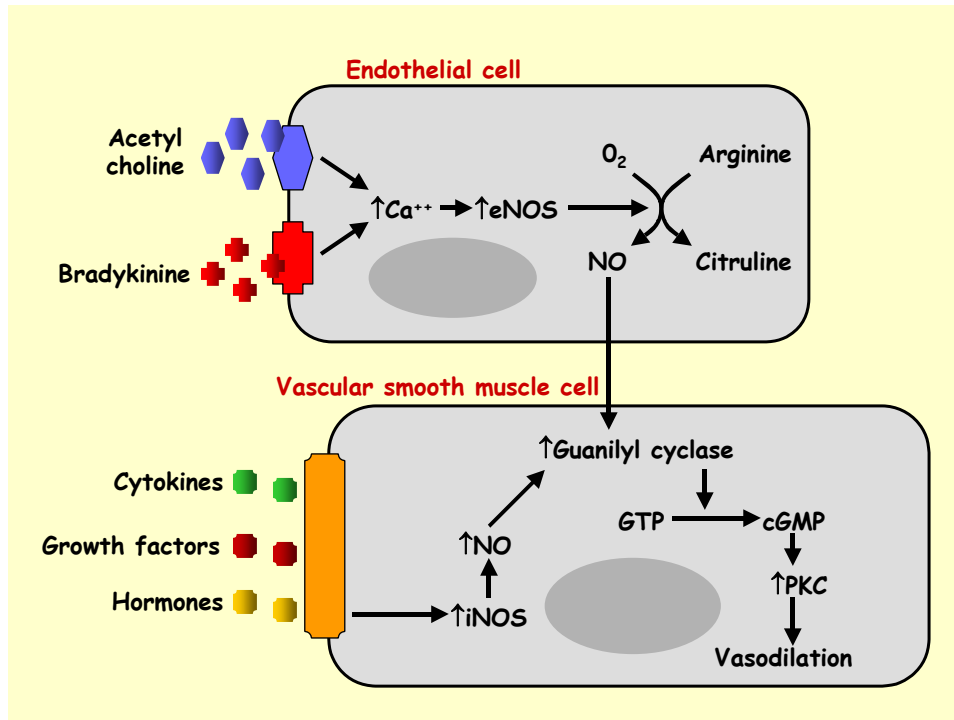


Figure 4.4. Nitric oxide signalling.

### Endothelial NOS

Endothelium-dependent vasodilation is the result of activation of the constitutive endothelial cell NOS (eNOS). Endothelium-dependent relaxation occurs in response to a wide variety of stimuli including acetyl choline, bradykinin, substance P, thrombin and adenine nucleotides. Binding of these ligands to their receptor leads to an influx of  $\text{Ca}^{++}$  and eNOS is activated by the increased  $\text{Ca}^{++}$  concentration (Fig. 1.10). [Knowles RG and Moncada S, 1992] The calmodulin/ $\text{Ca}^{++}$  complex is involved in this process and directly activates eNOS. Nitric oxide, being a gas, is not contained to the cytoplasm but freely disperses, without the need for carrier proteins or receptor, to surrounding cells including vascular smooth muscle cells. The NO then stimulates guanylyl cyclase that converts GTP to cGMP, which in turn leads to relaxation of the vascular smooth muscle cell. [Mancini L and Brandi ML, 1999]

### *Induced NOS*

Cytokine-induced vasodilation differs in that the activation of NOS involved is not dependent on the concentration of  $Ca^{++}$ . This process is mediated by a distinctly different and inducible isoform of NOS (iNOS) which in turn is induced by a variety of cytokines and endotoxin. Binding of these ligands to their receptors leads to an induction of iNOS and increased production of iNOS mRNA. The consequent increase in NO production leads to the same increase in cGMP and resultant vasodilation. Nitric oxide release also occurs in the central nervous system and at nerve ends and is the result of activation of another distinct NOS isoform, nNOS. The subsequent effects are the same as for the other systems. [Knowles RG and Moncada S, 1992]

It is therefore evident that NO mediates its effects by more than one means. On the one hand NO activates an enzyme, namely guanylyl cyclase. Enzymes other than NOS that are not directly involved in vascular biology may also be activated in a similar fashion. On the other hand NO can, as in the case of BMP-2, also induce the transcription of proteins. Not only is the NO system operative in the cells of the vasculature but it has become evident that this signalling system is also present in other cells and plays a role in other organ systems including bone.

### *NO and arterial health*

NO plays an important role in vascular physiology including the maintenance of vascular smooth muscle tone and many aspects of normal endothelial function. Abnormal endothelial function precipitated by various atherogenic insults is postulated to play an important role in atherogenesis. Nitric oxide has accordingly been stated to play a protective role in this respect. [Aengevaeren WR, 1999] Experimentally, an inhibition of NO production is associated with enhanced atherosclerosis that is reversed when the NO production is again normalised. [Boger RH, Bode-Boger SM et al., 1997]

The statins have also been observed to have a beneficial effect on atherosclerosis and its consequences, which cannot be explained solely by their effect on blood lipids and LDL-C. [Sessa WC, 2001] Numerous studies have demonstrated that statins increase eNOS activity, enhance the iNOS expression induced by cytokines and growth factors, and consequently increase NO production and the resultant effects thereof. [Chen H, Ikeda U et al., 2000a; Hernandez-Perera O, Perez-Sala D et al., 1998; Kaesemeyer WH, Caldwell RB et al., 1999; Laufs U, La Fata V et al., 1998; Mital S, Zhang X et al., 2000] Statins augment cerebral blood flow, reduce infarct size and neurological function when administered prophylactically in normocholesterolaemic mice. [Endres M, Laufs U et al., 1998] This effect prompted researchers, notably those from the Mundy group, to explore the effect of statins on bone and whether these did not also involve NO. [Garret IR, Esparza J et al., 2000; Garret IR, Chen D et al., 2001; Garrett IR, Gutierrez G, and Mundy GR, 2001] The effect of statins on eNOS expression is prevented if the cells are cultured in the presence of mevalonate or geranylgeranylpyrophosphate but not in the presence of farnesylpyrophosphate [Laufs U and Liao JK, 1998] indicating that prenylation is important, and suggesting that the process is mediated via Rho prenylation. The involvement of Rho prenylation was later proven by elegant studies showing that eNOS expression could be increased by the Rho inhibitor *Clostridium botulinum* C3 transferase and also by dominantly negative RhoA mutants, whereas eNOS expression could be decreased by *E. Coli* cytotoxic necrotising factor-1, an activator of Rho. [Laufs U and Liao JK, 1998; Laufs U, Gertz K et al., 2000] A major protective effect, or non lipid-modifying effect, of statins with respect to atherosclerosis has therefore been attributed to their enhancement of NO production.

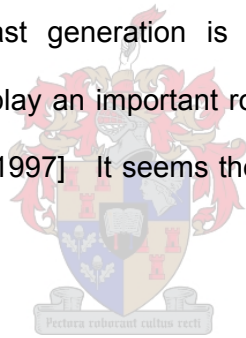
### *NO and the cytoskeleton*

There is a profound interaction between the elements of the cytoskeleton on the one hand and NOS activity and NO production on the other hand. The states of the actin microfilaments influence L-arginine transport and can thereby increase NO production. [Zharikov SI, Sigova AA et al., 2001] Inhibition of NO synthesis results in alterations of the endothelial cytoskeleton, which results in a venular leak of albumin. [Baldwin AL, Thurston G, and al NH, 1998] Inhibition of Rho, either by inhibition of prenylation with the statin mevastatin, or directly by *Clostridium botulinum* C3 exoenzyme, results in an enhanced iNOS activity and NO production evoked by the inflammatory cytokines. [Muniyappa R, Xu R et al., 2000] Therefore, Rho negatively regulates eNOS expression and activity. This effect of *Clostridium botulinum* exotoxin can be duplicated by statins via their inhibition of the geranylgeranylation of Rho that in turn inhibits Rho activity. [Laufs U and Liao JK, 1998; Hausding M, Witteck A et al., 2000] Statins therefore increase NOS activity and increase NO production. Indeed, withdrawal of statin treatment leads to a transient rise in Rho activity that results in an up to 90% reduction in NO production. [Laufs U, Endres M et al., 2000]

### *NO and osteoclasts*

The effects of NO are not limited to endothelial cells or vascular smooth muscle cells. The enhancement of NO production via increased eNOS or iNOS activity also extends to macrophages, those cells that play a pathogenic role in atherosclerosis. [Sumi D, Hayashi T et al., 2001; Chen H, Ikeda U et al., 2000b] In addition mesangial cells, which also belong to the macrophage lineage, are affected by statins in a similar manner to macrophages. [Chen H, Ikeda U et al., 2000b] Given these effects of NO on other cells of the macrophage lineage, it would be expected that osteoclasts would react in a similar fashion when exposed to NO.

Constitutive eNOS and iNOS have been identified in osteoclasts. [Alam AS, Huang CL et al., 1992] Together with  $\text{Ca}^{++}$ , NO has been identified as one of the local factors controlling osteoclastic resorption and it has been demonstrated to inhibit bone resorption. [Alam AS, Huang CL et al., 1992; Mancini L, Becherini L et al., 1997; Mancini L, Moradi-Bidhendi N et al., 1998; Mancini L and Brandi ML, 1999] Nitric oxide causes osteoclast detachment and contraction accompanied by a profound inhibition of bone resorption. [Brandi ML, Hukkanen M et al., 1995] [Dong SS, Williams JP et al., 1999] Others show that inhibition of NOS activity is associated with an increase in bone resorption. [Kasten TP, Collin-Osdoby P et al., 1994] Consequently it is postulated that NO maintains a central control of bone resorption by exerting a tonic restraint on osteoclast number and activity. [Brandi ML, Hukkanen M et al., 1995] Seemingly paradoxically, other researchers show that osteoclast generation is increased by cytokine-induced NO production from iNOS. NO may play an important role in certain pathologic conditions of bone. [Chae HJ, Park RK et al., 1997] It seems therefore that NO has a biphasic effect on the osteoclast.



#### *NO and osteoblasts*

Osteoblasts are also affected by NO. Marked abnormalities of postnatal bone formation are found in eNOS knockout mice; they display reduced bone formation and volume which is due to impaired osteoblast function and which can be restored by an exogenous NO donor. [Aguirre J, Buttery L et al., 2001] Mundy et al. have elegantly demonstrated that statins increase osteoblast activity via an increase of NO production. Unfortunately the origin of this increased production of NO has not been defined and could be from the osteoblasts themselves or from endothelial cells. The importance of NO in osteoblast differentiation has been demonstrated. [Afzal F, O'Shaughnessy M et al., 2000] These workers demonstrate that NO is required for proper differentiation and show that NO knockout mice have severe skeletal defects and that their osteoblasts have



impaired chemotaxis. Other workers demonstrate in eNOS knockout mice that eNOS and NO are essential for osteoblast development, maintenance of BMD and the response to estrogen after ovariectomy.

Osteoblasts themselves produce NO after stimulation by IL-1 alpha but not after exposure to other cytokines such as IL-1 beta, TNF-alpha or FN-gamma and also very little in the unstimulated state. Other researchers found that FN-gamma increased NO production by osteoblasts, and although IL-1 beta and TNF-alpha had a weak stimulatory effect on their own, they showed a strong synergy with NF-gamma. [Hukkanen M, Hughes FJ et al., 1995] Cytokine-stimulated NO production by cytokines can occur via iNOS as well as eNOS. [Gallagher ME, van't Hof RJ et al., 2002] This NO production by osteoblasts may play a role in the osteoblast-osteoclast interactions during inflammatory processes and the NO produced by osteoblasts acts as an important mediator of the effects of pro-inflammatory cytokines on bone. [Helfrich MH, Evans DE et al., 1997] Other researchers show that this cytokine-induced NO production by iNOS significantly suppresses osteoblast activity. [Hukkanen M, Hughes FJ et al., 1995] An animal model of inflammation-induced osteoporosis that is associated with increased levels of NO production by iNOS, when compared to controls, was characterised by increased numbers of osteoclasts and decreased numbers of osteoblasts. These deleterious effects in the inflammation-induced osteoporosis model could be reversed by the administration of a NOS inhibitor. [Armour KE, Van'T HR et al., 1999] It would appear therefore that NO has a variable effect on osteoclasts and osteoblasts depending on the amount of NO present, and biphasic responses induced by NO have been documented in numerous cell systems including osteoclasts. [Calabrese EJ, 2001] This would be an explanation for the biphasic effect of bone formation and bone resorption in response to different doses of simvastatin documented by us.

In summary, it appears that both osteoblasts and osteoclasts low levels of NO are required for normal cell function and differentiation where high concentrations as found with the inflammatory response give rise to inhibition of cell activity and formation. [Brandi ML, 1999]

It is of note that apart from the inflammatory cytokines, oestrogens and mechanical stress also stimulate NO production and this undoubtedly contributes to their effect on bone metabolism. [Ralston SH, 1997] Given the strong influence of statins on NO production and the important effect that NO has on osteoclast and osteoblast function, NO might be the final common pathway for the effect of statins on bone. It is also quite possible that certain levels of NO production are able to override the suppressive effect of Rho inhibition on cell growth. It is clear that NO is an important molecule that mediates many effects on bone and its constituents and may therefore play a role in the genesis of various processes leading to osteopenia.

#### **4.7.4. Inhibition of Rab proteins by Statins**

The effect of statins on bone might involve the inhibition of prenylation of other proteins including Rab. As has been mentioned before, an aminobisphosphonate has been developed which inhibits Rab prenylation selectively via the inhibition of geranylgeranyl transferase II. [Coxon FP, Helfrich MH et al., 2001; Coxon FP, Dunford JE et al., 2001] Rab prenylation is therefore important for the function of bone cells. It has previously been shown that statins inhibit prenylation of Rab and it is quite possible that a similar process might be involved in the statin effect on bone.

#### **4.7.5. Integrins**

As alluded to earlier, the effect of statins on bone might involve a process that has nothing to do with prenylation. In the introduction the importance of integrins in the activation of polarised and motile cells including osteoclasts was emphasised. [Burrige K

and Chrzanowska WM, 1996] The blocking of the  $\alpha(v)\beta3$  integrin of osteoclasts by the snake venom echistatin, which is an RGD containing disintegrin, prevents fusion of, as well as the function of, osteoclasts. [Nakamura I, Tanaka H et al., 1998] Antisense oligodeoxynucleotide targeted against an integrin gene suppresses osteoclast function. [Villanova I, Townsend PA et al., 1999] It is clear therefore that integrin function is important for osteoclast activity. Lovastatin has been shown to bind directly to a regulatory domain of leukocyte functional antigen, LFA-1, and consequently to prevent the conformational change of the integrin on binding with its ligand. [Frenette PS, 2001; Kallen J, Welzenbach K, and Ramage P, 1999; Weitz-Schmidt G, Welzenbach K, and Brinkmann V, 2001] It is therefore quite plausible that statins might have a similar non-prenylation-related effect on an osteoclast integrin.

#### **4.7.6. The effect of lipids on bone health**

It is known that statins have effects on the cardiovascular system that cannot be explained solely by their cholesterol-lowering effect, an effect referred to as the "pleiotropic effect of the statins". [Bellosta S, Bernini F et al., 1998] Because statin use is always associated with a reduction in cholesterol, it is difficult to divorce the cholesterol lowering effect from the pleiotropic effects experimentally. Although there are plausible biomolecular mechanisms by which statins could affect bone cells, the question arises whether the effects of statins on bone could be mediated via a lowering of cholesterol per se. That this might be so was suggested some years ago. [Wang GJ, Chung KC, and Shen WJ, 1995] These researchers investigated the effect of lipid clearing agents on steroid-induced osteoporosis in rabbits. They found that the statin lovastatin was able to prevent the steroid-induced bone loss of the femoral head of these animals. They were also able to demonstrate this effect for bezafibrate. The fibrates had at that stage not been shown to share any of the pleiotropic effects of the statins. These studies were performed before the ideas surrounding the pleiotropic effects of statins had been formulated and

before peroxisome proliferation activated receptors (PPAR), nuclear receptors, were discovered. Superficially, the assumption at the time must therefore have been made that these bone sparing effects were mediated directly via an alteration of the serum lipid levels. However, it is now known that the fibrates, including bezafibrate, are PPAR- $\alpha$  agonists and able to bind and activate this receptor. In addition it has now been established that the statin-mediated inhibition of Rho is also able to activate PPAR- $\alpha$ . [Martin G, Duez H et al., 2001] Therefore these drugs do partially share a common pathway involving Rho, which may be a plausible explanation for their effect on bone. Nonetheless, the question must be asked whether there are any biomolecular mechanisms by which an alteration in serum cholesterol levels could affect bone turnover or BMD.

#### *Links between lipids and bone health*

There are numerous links between atherosclerosis, dyslipidaemia [Parhami F, Morrow AD et al., 1997] and osteopenia. [Barengolts EI, Berman M et al., 1998; Hak AE, Pols HA et al., 2000; Jie KG, Bots ML et al., 1996; Stulc T, Ceska R et al., 2000] Osteoporosis and atherosclerosis both increasingly occur in advanced years of life. The occurrence of osteoporosis and atherosclerosis in the same age group suggests that these two conditions may share pathogenic factors. Osteoporosis is associated with atherosclerosis and vascular calcification [Boukhris R and Becker KL, 1972; Barengolts EI, Berman M et al., 1998; Hak AE, Pols HA et al., 2000] and this association has also been noted in South Africa. [Dent CE, Engelbrecht HE, and Godfrey RC, 1968] There is also an association between osteoporosis and various risk factors for atherosclerosis. [Broulik PD and Kapitola J, 1993] This association was thought to be purely due to age but other researchers have been able to demonstrate this association even when adjusting for age [Boukhris R and Becker KL, 1972] while others have not been able to confirm this. [Vogt MT, San Valentin R et al., 1997] Women with osteoporosis have a

greater risk for atherosclerosis than age-matched controls. [von der Recke P, Hansen MA, and Hassager C, 1999] Patients with osteoporosis also have more severe atherosclerosis and higher lipid levels, [Barengolts EI, Berman M et al., 1998] and have a greater risk for stroke death. [Uyama O, Yoshimoto Y et al., 1997] It has been demonstrated that high lipid levels inhibit osteoblastic differentiation and that hyperlipidaemia is associated with a reduced BMD in mice. [Demer LL, 2001] In humans a link between osteoporosis and lipid genotype has been established. [Hak AE, Pols HA et al., 2000] However, these findings have not been consistent and other workers have found that males with the most favourable lipid profiles have the lowest bone mineral density and those with the most atherogenic lipid profiles have the best BMD. [Adami S, Braga V et al., 2001b] Statins are known to be able to cause regression of atherosclerosis [Corsini A, Pazzucconi F et al., 1998] and this effect has also been demonstrated to occur with etidronate. [Zhu BQ, Sun YP et al., 1994] Furthermore, there is evidence that steroid-induced osteoporosis [Wang GJ, Chung KC, and Shen WJ, 1995] and other deleterious effects of steroids including suppression of osteoblast by steroids, and osteonecrosis, can be prevented by the use of statins. [Cui Q, Wang GJ et al., 1997]

*Similarities between bone and vascular tissue*[Adami S, Braga V, and Gatti D, 2001a; Braga V, Gatti D et al., 2001]

Bone and vascular tissue share many biomolecular and cellular features. [Parhami F, 2000] The endothelial cells, pre-osteoblasts and monocyte-derived osteoclasts found in bone have been shown to have counterparts in atherosclerotic lesions. Osteopontin, bone morphogenetic protein, matrix Gla protein, collagen I, osteonectin, osteocalcin, nitric oxide, and matrix vesicles are found in both bone and atherosclerotic lesions. Both atherosclerotic lesions and bone recruit monocytic cells that ultimately form foam cells and osteoclasts respectively. The arterial wall contains cells that can differentiate into

osteoblasts and go through the same stages of differentiation as bone osteoblasts and can produce bone mineral. [Parhami F, Morrow AD et al., 1997] Oxidised lipoproteins are known to promote atherosclerosis and are also able to induce mineralisation in the vessel wall. [Parhami F, Morrow AD et al., 1997; Towler DA, Bidder M et al., 1998] Somewhat paradoxically, these same oxidised lipids inhibit the osteoblastic differentiation of cells in bone. [Parhami F, Morrow AD et al., 1997]

Therefore various lines of evidence indicate that there seems to be more than a casual relationship between osteoporosis and atherosclerosis. It would therefore not be inconceivable that the treatment of one of the risk factors of atherosclerosis, namely dyslipidaemia, might have an impact on the bone health. In particular, the common origin of circulating monocytes and osteoclasts suggests a common reaction or response to drugs used to prevent either atherosclerosis or osteoporosis.

#### *Role of caveolae*

It was previously thought that the treatment for dyslipidaemia resulted in a change of the lipid composition of the cell membrane and that this in some way affected the behavior of the cell. However, no explanations were ever given as to the mechanisms by which these membrane changes could affect signal transduction pathways from the cell surface to the cytoplasm or even the nucleus. Research into this field has yielded insights into possible ways in which the treatment of dyslipidaemia could affect signalling within cells including the endothelium and possibly even bone cells.

Calmodulin and the protein caveolin play important roles in the regulation of NOS. [Kone BC, 2000] Cells, including endothelial cells, have small cholesterol-rich invaginations of the plasma membrane called caveolae which also contain large amounts of the protein caveolin. Caveolae have been demonstrated to play an important role in signal transduction and also a role in endothelial function through their association with

NOS and NO production. [Fielding CJ, 2001; Kinlay S, Libby P, and Ganz P, 2001] The caveolae contain and concentrate a number of signalling molecules in so-called lipid rafts; G protein-coupled receptors including muscarinic and bradykinin receptors, protein kinases, and the transmembrane protein, caveolin. Caveolae are also intimately related to the cytoskeleton, which may thus contribute to transduction of signals mediated by NO. Endothelial nitric oxide synthase binds caveolin or calmodulin in a mutually exclusive manner. Caveolin inhibits eNOS and calmodulin activates eNOS. In the resting state eNOS is bound to caveolin and eNOS is consequently suppressed. When  $Ca^{++}$  enters the cells it binds to and activates calmodulin, which then promotes a dissociation of eNOS from caveolin. The  $Ca^{++}$ /calmodulin complex then binds to eNOS and activates it. [Michel T and Feron O, 1997]

The cellular free cholesterol content also regulates the functions of caveolar proteins, including caveolin. [Fielding CJ, 2001] Hypercholesterolaemia and LDL cholesterol increase the synthesis of caveolin and its inhibitory binding to eNOS. [Feron O, Dessy C, and Moniotte S, 1999] Consequently it is not surprising to find a reduced production of NO in the presence of hypercholesterolaemia. In addition, oxidant stress may decrease the number of caveolae. [Peterson TE, Poppa V, and Ueba H, 1999] Statins have been shown to decrease caveolin expression and thereby to increase eNOS activation. [Feron O, Dessy C et al., 2001] However, LDL cholesterol reverses this direct effect of statins on eNOS, suggesting that the inhibition of caveolin expression results primarily from the reduction of LDL-cholesterol. [Davis ME and Harrison DG, 2001] These effects on caveolae might be a further mechanism by which statins could have an influence on NO production via an alteration of LDL-C, not only in endothelial cells but also other cells, and consequently also dictate their behavior and growth.

There is an additional mechanism by which altering the plasma cholesterol could affect cells. Sterol regulatory element binding proteins (SREBP) are membrane bound

transcription factors that regulate the transcription of HMG-CoA reductase and other genes. [Brown MS and Goldstein JL, 1997; Brown MS and Goldstein JL, 1998] SREBPs are released by a proteolytic mechanism that is regulated by the cellular sterol and cholesterol content, an effect that could therefore also be influenced by statins. SREBPs bind to sterol regulatory elements (SRE) and regulate the transcription of numerous gene products. These SREs play an important role in all cells including bone cells.

There are therefore numerous mechanisms for which supportive evidence is available by which statins could affect the behaviour of cells. It may well be that more than one mechanism may be operative under certain circumstances and it may also be that one mechanism will override another mechanism under other circumstances.

It has been proposed that the direct cholesterol-lowering effect of statins might play a role in the behaviour of bone cells. [Demer LL, ] However, very little in the way of biomolecular mechanisms are offered to explain this effect. Differences in the chow administered to our rats and those of Mundy et al could have resulted in different LDL-cholesterol levels in our animals that may then have affected bone cells differently. However, the work by Mundy et al appears to have been thorough and one must conclude that the explanation offered by them seems to be the most plausible.[Parhami F, 2000; Parhami F, Tintut Y et al., 2001]

#### **4.8. The biphasic effect**

We have been the first researchers to demonstrate a biphasic effect for statins on parameters of bone turnover. We have shown that the relatively large dose of simvastatin, 20mg/Kg/day, increased parameters of bone formation. Conversely, the much smaller dose of simvastatin, 1mg/Kg/day, inhibited bone formation when compared to controls. Of note is that these smaller dose of simvastatin resulted in an inhibition of parameters of bone formation and not merely a lesser increase - conceptually an important point.



Similarly, simvastatin 20mg/Kg/day produced an increase of the parameters of bone resorption whereas smaller doses of simvastatin resulted in a decrease in these resorptive parameters when compared to their controls - again, an inhibition of resorption rather than merely a lesser increase. At the smallest dose of simvastatin, 1mg/Kg/day, the parameters of bone resorption again increased, resulting in a U-shaped curve.

This initially seemed to be without precedent. However, other workers, while researching the effect of a bisphosphonate, EB-1053, on osteoclast function have unwittingly recorded a similar biphasic effect. The researchers concluded in their article that overall, this bisphosphonate inhibited osteoclast function. However, in the series of dosages that they tested, it is recorded that the smallest dose resulted in an increase in osteoclast function. [van der Pluijm G, Binderup L et al., 1992] Unfortunately no further comment is made by the authors regarding this phenomenon. It seems more than coincidental that two different prenylation inhibitors, a statin and a bisphosphonate, result in similar biphasic response.

#### **4.8.1. Multiple signalling pathways**

The signalling pathways involved in the activation of polarised and motile cells are complex and multiple. (Fig. 1.9.) [Denhardt DT, 1996] Amongst others, they involve multiple receptors, various second messengers, re-arrangement of the cytoskeleton and induction of growth factors such as BMP-2. In addition there is a substantial amount of cross-talk between the different signalling cascades. It is therefore conceivable that signalling down one pathway can be overridden in a dose-dependant fashion by signalling down another pathway that has an opposing effect. Indeed, this seems to be what is happening. Theoretically, if the prenylation of Rho is inhibited then this should lead to diminished Rho activity, which in turn should led to reduced osteoblast activity. However, this diminished Rho activity actually leads to the activation of the NO and BMP-2 pathways which then stimulate the osteoblast.

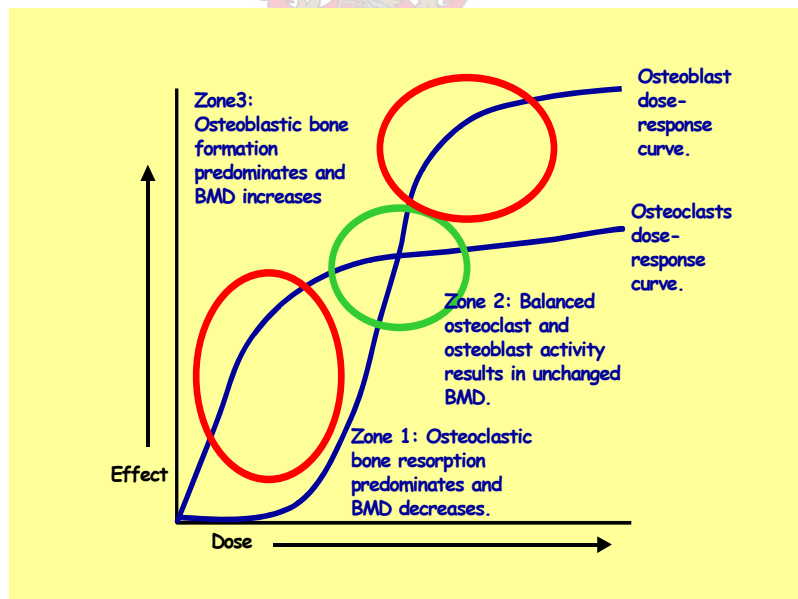
#### **4.8.2. The biphasic effect of NO signalling**

Research into NO signalling, including its effects on bone, has also offered another plausible explanation of the biphasic effect of statins on parameters of bone turnover. Cytokines combined with IFN-gamma result in a superinduction of NO synthesis that is largely responsible for the selective inhibitory effect of IFN-gamma on cytokine-induced bone resorption. [Evans DM and Ralston SH, 1996] These high concentrations of NO are also inhibitory to osteoblasts and are partly responsible for the inhibitory action of cytokines on osteoblast proliferation. However, at lower doses the NO has different effects; moderate induction of NO increases bone resorption and promotes the proliferation of osteoblasts. [Evans DM and Ralston SH, 1996] The bi-directional nature of NO signalling in the osteoclast has also been demonstrated; a basal production of NO is required for osteoclast differentiation while at higher doses osteoclast activity is inhibited. [Mancini L, Becherini L et al., 1997] These biphasic effects of cytokine-induced NO production have been demonstrated by others. [Ralston SH, Ho LP et al., 1995; Ralston SH, 1997] Mundy et al. have clearly shown that NO is involved in the effect of statins on bone. It is equally conceivable that different doses statin will produce different rates of production of NO and hence a biphasic effect as described above. Therefore the biphasic response noted by us has a plausible explanation which is based on sound research and further supports our findings.

It is also clear that we did not study the smallest non-effective dose of simvastatin and did not follow the dose response curve back to where there would be not effect on bone. Had we done so we would have demonstrated a U-shaped dose response curve for BMD. Clearly these finding require an explanation.

#### 4.8.3. Biphasic effect from signalling pathways with differing dose-response curves

The presence of a U-shape dose-response curve implies that there are two different processes or signalling pathways, with different sensitivities, operative. In fact the BMD is the cumulative effect of two processes, namely bone formation and bone resorption. If the dose-response curves of these two processes differed, as indeed they have been demonstrated by us to do, then it is conceivable that one process could start working before the other has had time to exert its effect. We have clearly demonstrated that the dose response curves of bone formation and bone resorption differ from each other. It has also been demonstrated that different cell types have different sensitivities to statins. [Newman A, Clutterbuck RD et al., 1994] Accordingly, if osteoclasts were to be more sensitive to simvastatin then bone resorption would be the first to be stimulated at small doses of simvastatin (Figs. 4.5.).



**Figure 4.5. The effect of differing dose response curves of bone turnover on BMD.**

At higher doses osteoblasts would also be stimulated and bone resorption would start balancing the effect of resorption. A dose would then be reached where resorption and formation are equally active and balance each other, resulting in no change in the

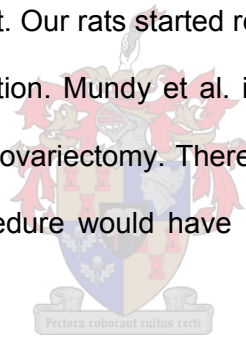
BMD. At higher dose still, a zone could be reached where formation outstrips resorption and the BMD would increase. This model fits and explains our data very well. Clearly the dose-response curve for simvastatin on BMD is the cumulative effect of the dose response of resorption and of formation and cannot logically be explained in any other way. Indeed this is the first time that this conceptual model has been used to explain the dose responses for the effect of drugs on BMD. There is no reason to believe that there will not be similar dosage effects in humans and a similar model would be important to interpret findings at a clinical level.

#### **4.9. Possible reasons for differences in results between studies.**

We think that there are not many, but certainly fundamental as they pertain to resorption, differences between the findings of our studies and those of Mundy et al. In both our studies simvastatin at doses of 10mg - 20mg were investigated and in both our studies an increase in parameters of bone formation were found. Therefore in this respect the results of the studies are not contradictory. Regarding bone formation, one of the differences between our studies is that we found a decrease in bone formation at the lesser doses of simvastatin whereas Mundy et al did not. However, Mundy et al. did not report any data relating to simvastatin at the very low doses used i.e. 1mg/Kg/day. Therefore our findings at these low doses are not strictly comparable with the findings of Mundy et al who report on the effect of higher doses. We also found varying results of bone resorption in response to different doses of simvastatin whereas Mundy et al. only state that bone resorption was decreased and provide very little in the way of data to illustrate this. Herein might be a further point of difference but again, the data is not truly comparable. There might be other reasons why these differences have been observed between our data and that of Mundy et al.

#### **4.9.1. Differences in experimental animals**

The first factor, which might have made a difference between our studies, is the animals used in the studies. Mundy et al used male Swiss ICR white mice for their calvarial studies. In their studies exploring the effect of systemic simvastatin via oral gavage they used rats but do not comment on the type of rats used. We used female Sprague Dawley rats throughout our studies. Due to differences in size it would be expected that the metabolism of the animals will be different but unfortunately no literature could be found which directly compares the bone metabolism of mice and rats. Otherwise the age of the rats and the timing of the ovariectomy between the studies were not markedly different. The age of the rats in our studies and those of Mundy et al. was three months and therefore not different. Our rats started receiving simvastatin within 10 days of their ovariectomy or sham operation. Mundy et al. included groups of rats that received simvastatin within 7 days of their ovariectomy. Therefore it is unlikely that the type rats or the timing of the operative procedure would have made a difference to the findings in these studies.



#### **4.9.2. Duration of treatment**

The duration of treatment might have been important in explaining the differences in the results between the studies. Mundy et al administered the simvastatin orally for 35 days whereas our rats were given simvastatin orally for 56 or 84 days. Although no data could be found to indicate that the duration of treatment with a statin makes any difference in the ultimate effect produced by the statin, this is certainly a possibility.

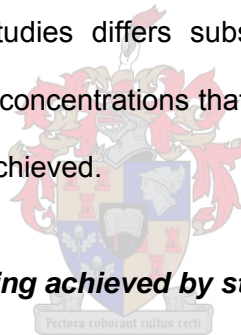
#### **4.9.3. Differences in bioavailability**

We convincingly showed that different doses of simvastatin produce different effects on parameters of bone turnover and also on BMD. In effect the only difference between the rats receiving the different doses of simvastatin is the amount of statin that reaches the blood/bone interface. Therefore any factor which affects the amount of statin that reaches the bone will conceivably also have an influence on the effect of the statin on bone. Consequently, statins that bypass the first pass extraction by the liver, either due to systemic administration such as dermal application, or because of pharmacokinetic properties such as hydrophilicity, will have different concentrations that reach the plasma/bone cell interface.

In our studies there were clear and marked differences in the parameters of bone turnover between the rats that received simvastatin versus the rats that served as controls and only received placebo. We can therefore categorically state that some unknown proportion of simvastatin was absorbed but we cannot be sure of the amount. We administered the simvastatin orally by dissolving it in vegetable oil and mixing it in the feeds of the animals. Feed supply was controlled to ensure that all drug was consumed every day. The first pass extraction of simvastatin by the liver exceeds 90% and therefore at best only 10% of the amount of simvastatin administered reached the systemic circulation. Furthermore, the admixture of the simvastatin with the vegetable oil and the feeds could further have reduced the bioavailability of the simvastatin so that the amount that ultimately reached the blood/bone interface could have been even less.

On the other hand, in their ex vivo calvarial experiments Mundy et al injected simvastatin into the subcutaneous tissue overlying the calvaria of their mice and therefore exposing the underlying bone to relatively large doses of simvastatin. This could have important consequences on the ultimate effect on the underlying bone. In reporting the

results of their later studies the actual method of administration of the statin by these researchers is not stated. [Garret IR, Esparza J et al., 2000; Garret IR, Chen D et al., 2001; Garrett IR, Gutierrez G, and Mundy GR, 2001] The group of Mundy has been looking at alternative methods for delivery of the statin to the bone interface. [Whang K, Zhao M et al., 2000] They have also developed an alternative method of administering the statin for these rat studies, namely by dermal application. [Gutierrez G, Garret IR et al., 2001] If this is the case then the amount of statin that reaches the blood/bone interface will be much higher than with oral administration. Dermal or subcutaneous administration bypasses the first-pass extraction by the liver and the amount of simvastatin that reaches the bone could be at least 10-fold higher than after oral administration. It may well be that the effective concentration of simvastatin at the plasma/bone interface in our studies differs substantially from that achieved in the experiments by Mundy et al - the concentrations that they achieve may be many orders of magnitude higher than what we achieved.



#### ***4.9.4. Differences in lipid-lowering achieved by statins***

If we are to believe that the plasma lipid or cholesterol concentration directly influence parameters of bone turnover [Parhami F, Jackson SM et al., 1999; Parhami F, 2000; Parhami F, Tintut Y et al., 2001] then it is possible that there could have been different lipid contents in the chow fed to the animals in the experiments by ourselves and those of Mundy. Accordingly this could have led to different cholesterol concentrations in the respective laboratory animal which could in turn have influenced the parameters of bone turnover. As pointed out in an earlier chapter, we do not think that this explanation is plausible.

#### 4.10. Effect of oestrogen

We have clearly shown that simvastatin has little effect on BMD and parameters of bone resorption and formation in ovariectomised animals. More importantly, our studies have made it evident that simvastatin at the doses we used was not able to prevent the loss of BMD and the other QBH features of the oestrogen-deprived state. (Study 3.1; Fig. 3.1.5). Unfortunately it does not seem as if Mundy et al. made this kind of comparison in their study. [Mundy G, Garrett R et al., 1999] Other researchers have also shown that simvastatin was unable to restore the bone loss after ovariectomy as determined by NMD and QBH. [Gallagher ME, van't Hof RJ et al., 2002; Solomon DH, Finkelstein JS et al., 2001; Yao W, Li CY et al., 2001] These findings suggest that oestrogen might play some permissive role in the action of statins on bone metabolism. However, an aminobisphosphonate and a potent inhibitor of prenylation, zoledronic acid, was able to inhibit all the negative effects in bone associated with estrogen deficiency in laboratory animals. [Green JR, 2001] Although statins may have caused a similar effect by causing hypogonadism, this has been excluded in our studies by the appropriate oestrogen and rFSH in the ovariectomised and intact rats.

The statins are able to induce a G1 phase cell cycle arrest by interfering with the mitogenic activity of wide range of cells including cancer cells. [Addeo R, Altucci L et al., 1996] When the culture medium of oestrogen-responsive MCF-7 breast cancer cells is augmented by oestrogen, then the cell cycle arrest induced by lovastatin or simvastatin does not occur and the HMG-Co reductase activity and the prenylation pattern are not affected. This effect of oestrogen can be blocked by steroidal and non-steroidal anti-oestrogens and also does not occur in oestrogen receptor negative cells. [Addeo R, Altucci L et al., 1996; Bonapace IM, Addeo R et al., 1996] In this instance oestrogen is needed, and is permissive for, this particular effect of statins.



Lastly, other researchers have also shown that simvastatin in doses ranging from 0.3 to 10mg/Kg/day and administered for 60 days failed to prevent osteopenia after ovariectomy. [Yao W, Li CY et al., 2001] This data supports our findings that statins do not protect against the osteopenia which occurs after ovariectomy.

#### **4.11. The effects of other statins.**

Most of the research done on bone with statins has involved the use of simvastatin. We have shown that two other statins, namely atorvastatin and pravastatin, also reduced BMD in our rat model. Lovastatin, mevastatin and fluvastatin have been investigated regarding their effect on QBH and have been shown to have the same results on bone as simvastatin. [Mundy G, Garrett R et al., 1999] This is not entirely surprising as these statins used by Mundy and co-workers all have similar pharmacokinetic properties and bioavailabilities i.e. their absorption, half-lives, first pass extraction by liver, lipid-solubility and hence their volume of distribution are similar.

However, as stated in the preamble to Studies 3.4 and 3.5, there is reason to believe that statins might not all behave in the same way. The statins all inhibit the cholesterol synthetic pathway via inhibition of hydroxymethylglutaryl CoA reductase but do have other effects which are often assumed to be shared by all via an assumed class effect. The chemical formulae of the statins differ markedly and they are often divided into the "natural" and "synthetic" statins" where simvastatin and pravastatin are classed as "natural" and atorvastatin classed as "synthetic. [Rosenson RS and Tangney CC, 1998] These dissimilarities may affect the way that they bind to target molecules other than hydroxymethylglutaryl CoA reductase, and consequently engender different properties to the various statins.

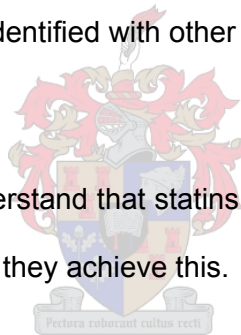
The metabolism, bioavailability and consequently the amount of drug that reaches the bone/plasma interface differs between the statins. We have already demonstrated the

importance of the dosage of statin in determining the effect seen by that statin. The lipid soluble statins such simvastatin and atorvastatin will have a high first pass extraction by the liver, in the region of >90%. Conversely they easily cross the membranes of peripheral cells and therefore the small amount statin remaining after passing through the liver does not have much difficulty in penetrating and affecting peripheral cells. Pravastatin is hydrophilic and therefore does not cross membranes easily and consequently the first pass extraction by the liver is only in the region of 60%. The larger amount of statin remaining after passage through the liver finds it relatively difficult, because of its hydrophilic characteristics, to cross the membranes of peripheral cells to exert an effect. [Corsini A, Bellosta S et al., 1999a; Desager JP and Horsmans Y, 1996]

The half-life of most statins is in the order of 2 hours whereas atorvastatin has a half-life exceeding 18 hours. [Posvar EL, Radulovic LL et al., 1996; Cilla DD, Whitfield LR et al., 1996; Desager JP and Horsmans Y, 1996] The administration of atorvastatin therefore results in continuously raised blood levels of the drug during the course of a 24 hour day with no dips in the drugs levels; consequently cells are continuously exposed to the effect of the statin. This may be one of the reasons for the cholesterol-lowering potency of the drug. The other statins have therapeutic levels for only part of the day and there are long periods when cells are not under the influence of these drugs. The use of atorvastatin therefore amounts to continuous dosing, compared to micro-intermittent dosing with the use of the other statins. Differences in the effect of parathyroid hormone on bone have been noted when continuous dosing is used compared to intermittent dosing. Continuous dosing with PTH results in osteopenia whereas intermittent dosing with PTH is associated with an increase in bone mineral density. [Masiukiewicz US and Insogna KL, 1998] Bearing in mind the number of important signalling systems that are impinged upon by the statins, particularly those utilising prenylated proteins which play a pivotal role in cell growth, differentiation and activation of cells, these differences in half-life may have important consequences in different organ systems.

We have found that atorvastatin potently reduces BMD in our rat model. Although the studies were not designed to directly compare the effect of different types statins on BMD, the tests with the different equipotent cholesterol-lowering doses of atorvastatin, pravastatin and simvastatin were performed simultaneously and a comparison of the effect seen with different statins would not be totally invalid. The reduction of BMD seen with atorvastatin 2,5mg/Kg/day was of a much greater magnitude than the equipotent cholesterol-lowering dose of simvastatin 5mg/Kg/day. This suggests that there might be some differences in the way that these two statins affect bone. Atorvastatin has a greater effect on increasing fibrinogen levels when compared with simvastatin which is neutral. [Song JC and White CM, 2001; Rosenson RS and Tangney CC, 1998] It has been shown that atorvastatin use leads to tachyphylaxis which has been ascribed to its long half-life and which has not been identified with other statins. [Cromwell WC and Ziajka PE, ]

It is therefore easy to understand that statins may differ in their effect on bones, as well as the mechanisms by which they achieve this.



## Chapter 5: Conclusions and future directions

### 5.1. Conclusions.

The following conclusions can be made from the studies

- Statins affect bone and mineral metabolism
- Statins, under certain circumstances, decrease BMD
- The effect of simvastatin on parameters of bone turnover is dose-dependant
- Simvastatin increases parameters of formation at higher doses
- Simvastatin decreases parameters of bone formation at lower doses
- Simvastatin increases parameters of bone resorption at higher doses
- Simvastatin decreases parameters of bone resorption at lower doses
- Dose response curves of simvastatin for parameters of bone formation and bone resorption differ
- Statins have very little effect in the absence of estrogen
- Statins are not able to prevent post-ovariectomy osteopaenia
- Statins, other than simvastatin, namely atorvastatin and pravastatin, also reduce BMD.

### 5.2. Future directions.

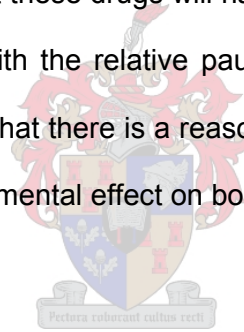
- A wider range of doses for simvastatin must be investigated i.e. smaller doses going back to a dose where there is no effect of the statin must be obtained. In

other words, a complete dose response curve for simvastatin should be obtained. Only in that way can we get a better picture of what happens to bone at different doses of the simvastatin.

- The effect of different times of statin exposure must be investigated. It may be that the effect early during the exposure to the statin is different from effect obtained later during exposure. For a full understanding of the effect of statins on parameters of bone turnover and for the interpretation of different studies, it will be important to know whether the effect seen after a short time exposure is the same as a long exposure. The exposure time from 2 weeks to 12 weeks is suggested.
- The experiments that have been done for simvastatin must also be repeated for other statins to determine the different ways that they affect bone health.
- The effect of statins on bone in the presence and absence of estrogen must be investigated in more detail to determine what the interaction is between oestrogen and statins.
- It is assumed that all the effect of the statins on bone are via the inhibition of prenylation and this has been confirmed by Mundy and his co-workers. However, there is a possibility that there may be more than one mechanism operative simultaneously. It would therefore be of use to see if the administration of farnesol or geranylgeraniol *in vivo* would be able to totally prevent the effects of statins on bone. Furthermore it would be important to see whether NO inhibitors *in vivo* can totally inhibit the effects of statins on bone and thereby also determine whether there are not other mechanisms by which statins have their effect on bones.

- Prospective studies using statins in humans with BMD and biochemical markers of bone turnover as endpoints are required to see what the effects of statins are on bone health in humans. Simultaneous QBH would be of great help but this is an invasive procedure and might not be acceptable to a large number of people.

It is evident that statins have an effect on bone in laboratory animals and it is also clear that the effect of statins on bone in laboratory animals may under certain circumstances be detrimental. It is therefore important that further research be done to determine the extent of the effect of statins on bone both in the laboratory animals and humans. In the meantime, an automatic assumption that statins will increase BMD cannot be made and the assumption that these drugs will have a beneficial effect in the treatment of osteoporosis is not warranted with the relative paucity of information available. Indeed, the available evidence suggests that there is a reasonable chance that statins may, under certain circumstances, have a detrimental effect on bone health.



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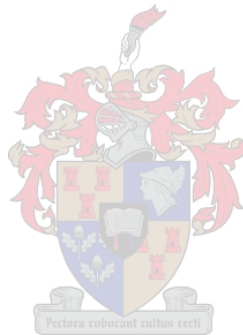
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Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

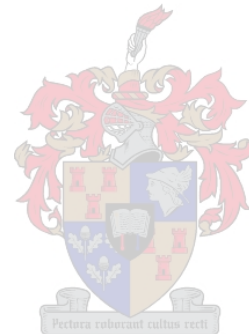
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Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Bone Mineral Density: Descriptive statistics

Descriptive Statistics : BMD Study 3.1

Group	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Standard		Skewness	Kurtosis
			-95.000%	+95.000%					Std.Dev.	Error		
Sh	10	0.10367	0.099197	0.108143	0.10380	0.0927	0.1103	0.000039	0.006253	0.001977	-0.779627	-0.431294
Sh-S	10	0.09935	0.094234	0.104466	0.10135	0.0899	0.1111	0.000051	0.007152	0.002262	0.068319	-1.299149
OVX	10	0.09380	0.089380	0.098220	0.09230	0.0855	0.1044	0.000038	0.006178	0.001954	0.646418	-0.374060
OVX-S	10	0.09374	0.088947	0.098533	0.09365	0.0844	0.1045	0.000045	0.006701	0.002119	0.127230	-1.112783

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Bone Mineral Density: Statistical analyses - Mann Whitney U-test

**Mann-Whitney U Test: BMD Study 3.1**

By variable GROUPS

Group 1: 100-Sh Group 2: 102-OVX

	Rank Sum	Rank Sum	U	Z	p-level	adjusted	p-level	Valid N	Valid N	2*1sided
	Sh	OVX								
BMD Study 3.1	141	69	14	2.7213	0.0065	2.7224	0.0065	10	10	0.0052

**Mann-Whitney U Test: BMD Study 3.1**

By variable GROUPS

Group 1: 100-Sh Group 2: 101-Sh-S

	Rank Sum	Rank Sum	U	Z	p-level	adjusted	p-level	Valid N	Valid N	2*1sided
	Sh	Sh-S								
BMD Study 3.1	122	88	33	1.2851	0.1988	1.2856	0.1986	10	10	0.2176

**Mann-Whitney U Test: BMD Study 3.1**

By variable GROUPS

Group 1: 102-OVX Group 2: 103-OVX-S

	Rank Sum	Rank Sum	U	Z	p-level	adjusted	p-level	Valid N	Valid N	2*1sided
	OVX	OVX-S								
BMD Study 3.1	105	105	50	0.0000	1.0000	0.0000	1.0000	10	10	1.0295

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Bone Mineral Density: *Statistical analyses - ANOVA; All effects*

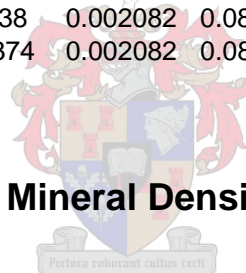
#### Study 3.1: LS Means (anova)

Current effect: F(3, 36)=5.3264, **p=.00385**

Effective hypothesis decomposition

	Group	BMD Mean	BMD Std.Err.	BMD -95.00%	BMD +95.00%	N
1	Sh	0.10367	0.002082	0.099448	0.107892	10
2	Sh-S	0.09935	0.002082	0.095128	0.103572	10
3	OVX	0.0938	0.002082	0.089578	0.098022	10
4	OVX-S	0.09374	0.002082	0.089518	0.097962	10

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Bone Mineral Density: *Statistical analyses - ANOVA; Differences between groups*



#### Fisher's LSD test; variable BMD

Probabilities for Post Hoc Tests

Error: Between MS = .00004, df = 36.000

	Group	{1}	{2}	{3}	{4}
1	Sh	.10367	.09935	.09380	.09374
2	Sh-S	0.150935	0.150935	0.067484	0.064705
3	OVX	<b>0.001893</b>	0.067484	0.983852	0.983852
4	OVX-S	<b>0.00179</b>	0.064705	0.983852	0.983852

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: *Descriptive statistics*

#### Descriptive Statistics

Sh	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Std.Dev.	Standard		
			-95.000%	+95.000%						Error	Skewness	Kurtosis
Bone Volume (BV/TV)(%)	9	18.015	15.605	20.425	18.028	13.083	23.528	9.830	3.135	1.045	0.223	0.005
Osteoid volume OV/BV (%)	9	0.801	0.204	1.398	0.547	0.154	2.760	0.603	0.777	0.259	2.429	6.448
Osteoid volume OV/TV (%)	9	0.130	0.057	0.204	0.089	0.028	0.361	0.009	0.096	0.032	1.960	4.673
Osteoid Surface OS/BS (%)	9	4.408	1.824	6.992	3.507	1.847	12.806	11.298	3.361	1.120	2.344	6.061
Osteoblast surface Ob.S/BS (%)	9	0.492	0.239	0.744	0.379	0.107	1.048	0.108	0.329	0.110	0.753	-0.836
Osteoid thickness O.Th (mcm)	9	7.660	5.407	9.914	7.556	3.778	13.600	8.597	2.932	0.977	0.730	1.304
Eroded surface ES/BS (%)	9	6.055	3.880	8.230	5.348	3.325	12.272	8.008	2.830	0.943	1.498	2.275
Osteoclast surface Oc.S/BS (%)	9	0.736	0.448	1.024	0.616	0.369	1.567	0.140	0.374	0.125	1.537	2.480
Osteoclast number N.Oc/T.A. (/mm <sup>2</sup> )	9	0.057	0.035	0.079	0.045	0.033	0.121	0.001	0.028	0.009	1.735	2.809
Mineralising surface MS/BS (%)	9	5.105	3.300	6.909	5.337	2.323	9.922	5.512	2.348	0.783	0.896	1.158
Osteoid apposition rate OAR xi	9	0.855	0.623	1.087	0.798	0.603	1.595	0.091	0.302	0.101	2.172	5.418
Mineralisation lag time Mlt (days)	9	0.593	0.319	0.867	0.524	0.250	1.385	0.127	0.356	0.119	1.575	2.646
Bone formation rate BFR/BS (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	9	15.450	9.632	21.267	14.932	5.260	32.099	57.283	7.569	2.523	1.212	2.743
Rel mineral Vol	9	99.199	98.602	99.796	99.453	97.240	99.846	0.603	0.777	0.259	-2.429	6.448
Surface Density	9	5.140	4.635	5.644	5.299	4.398	6.425	0.431	0.656	0.219	0.686	0.395
Resting Surface	9	89.537	85.731	93.344	91.133	78.696	94.704	24.523	4.952	1.651	-1.629	2.326
Surf dens ost seams	9	0.220	0.108	0.331	0.192	0.083	0.572	0.021	0.145	0.048	2.026	5.077
Surf dens ostoid osteoblast interface	9	0.025	0.012	0.039	0.021	0.005	0.059	0.000	0.018	0.006	1.029	0.203
Ostoid thickness index	9	17.231	12.342	22.120	18.383	8.004	29.156	40.455	6.360	2.120	0.470	0.226
Surface density of Howship's lacunae	9	0.322	0.174	0.469	0.260	0.146	0.788	0.037	0.192	0.064	2.073	5.167
Surface density of bone osteoclast interface	9	0.039	0.020	0.059	0.031	0.017	0.101	0.001	0.025	0.008	2.119	5.219
Total osteoclasts (v)	9	0.302	0.155	0.449	0.240	0.180	0.775	0.037	0.191	0.064	2.280	5.598
Bone osteoclasts (TRS)	9	0.972	0.757	1.188	0.949	0.670	1.415	0.079	0.280	0.093	0.461	-1.425
Fractional labeled surfaces	9	8.820	5.689	11.951	8.989	4.156	17.493	16.589	4.073	1.358	1.094	1.810
Fractional double labeled surfaces	9	1.389	0.761	2.017	1.493	0.253	2.614	0.667	0.817	0.272	0.004	-1.023

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: Descriptive statistics

#### Descriptive Statistics

Sh-St	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Std.Dev.	Standard		
			-95.000%	+95.000%						Error	Skewness	Kurtosis
Bone Volume (BV/TV)(%)	10	17.286	14.374	20.198	17.887	10.667	23.778	16.567	4.070	1.287	-0.364	-0.275
Osteoid volume OV/BV (%)	10	1.554	0.884	2.223	1.351	0.306	3.359	0.876	0.936	0.296	0.865	0.324
Osteoid volume OV/TV (%)	10	0.265	0.141	0.388	0.210	0.056	0.556	0.030	0.172	0.055	0.952	-0.350
Osteoid Surface OS/BS (%)	10	9.534	6.418	12.649	9.171	2.798	16.406	18.968	4.355	1.377	-0.049	-0.730
Osteoblast surface Ob.S/BS (%)	10	1.117	0.748	1.486	1.152	0.000	1.995	0.266	0.516	0.163	-0.693	2.352
Osteoid thickness O.Th (mcm)	10	7.053	5.680	8.426	7.212	4.000	10.524	3.684	1.919	0.607	0.214	0.096
Eroded surface ES/BS (%)	10	8.110	6.566	9.653	8.259	4.297	12.718	4.657	2.158	0.682	0.512	2.448
Osteoclast surface Oc.S/BS (%)	10	1.205	0.898	1.512	1.217	0.427	1.869	0.184	0.429	0.136	-0.308	-0.145
Osteoclast number N.Oc/T.A. (/mm <sup>2</sup> )	10	0.109	0.084	0.134	0.109	0.049	0.170	0.001	0.035	0.011	0.015	-0.024
Mineralising surface MS/BS (%)	10	5.183	3.541	6.825	5.228	2.451	9.669	5.269	2.295	0.726	0.668	0.128
Osteoid apposition rate OAR xi	10	0.825	0.696	0.953	0.792	0.576	1.197	0.032	0.180	0.057	0.887	0.829
Mineralisation lag time Mlt (days)	10	0.541	0.357	0.726	0.475	0.241	0.981	0.067	0.258	0.082	0.621	-0.947
Bone formation rate BFR/BS (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	10	15.650	10.637	20.663	17.092	5.246	25.612	49.104	7.007	2.216	-0.222	-1.431
Rel mineral Vol	10	98.446	97.777	99.116	98.649	96.641	99.694	0.876	0.936	0.296	-0.865	0.324
Surface Density	10	4.915	4.120	5.709	4.756	3.424	6.318	1.233	1.110	0.351	0.073	-1.796
Resting Surface	10	82.357	79.068	85.646	82.290	74.896	89.218	21.137	4.598	1.454	-0.083	-0.500
Surf dens ost seams	10	0.445	0.303	0.587	0.397	0.177	0.775	0.039	0.199	0.063	0.549	-0.849
Surf dens ostoid osteoblast interface	10	0.054	0.031	0.076	0.052	0.000	0.125	0.001	0.031	0.010	0.910	3.543
Ostoid thickness index	10	15.651	12.978	18.325	14.790	10.945	22.807	13.965	3.737	1.182	0.774	0.126
Surface density of Howship's lacunae	10	0.410	0.283	0.536	0.397	0.173	0.796	0.031	0.177	0.056	0.951	1.739
Surface density of bone osteoclast interface	10	0.061	0.040	0.081	0.054	0.018	0.099	0.001	0.028	0.009	0.029	-1.521
Total osteoclasts (v)	10	0.549	0.378	0.719	0.496	0.202	0.901	0.057	0.238	0.075	0.095	-1.401
Bone osteoclasts (TRS)	10	1.476	0.990	1.963	1.547	0.559	2.625	0.462	0.680	0.215	0.278	-0.552
Fractional labeled surfaces	10	8.180	5.530	10.829	7.701	3.905	14.758	13.719	3.704	1.171	0.659	-0.609
Fractional double labeled surfaces	10	2.186	1.364	3.008	2.175	0.735	4.580	1.321	1.149	0.363	0.719	0.948

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: Descriptive statistics

#### Descriptive Statistics

OVX	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Std.Dev.	Standard		
			-95.000%	+95.000%						Error	Skewness	Kurtosis
Bone Volume (BV/TV)(%)	9	10.540	8.507	12.574	9.917	7.850	15.342	6.998	2.645	0.882	1.096	-0.031
Osteoid volume OV/BV (%)	9	2.324	1.377	3.271	1.931	0.836	4.878	1.518	1.232	0.411	1.134	1.206
Osteoid volume OV/TV (%)	9	0.229	0.151	0.307	0.217	0.128	0.444	0.010	0.102	0.034	1.318	1.667
Osteoid Surface OS/BS (%)	9	11.535	7.620	15.450	10.795	5.986	18.904	25.938	5.093	1.698	0.501	-1.582
Osteoblast surface Ob.S/BS (%)	9	0.773	0.329	1.217	1.100	0.000	1.408	0.333	0.577	0.192	-0.472	-1.817
Osteoid thickness O.Th (mcm)	9	9.608	6.885	12.330	9.951	5.368	13.949	12.546	3.542	1.181	-0.062	-2.006
Eroded surface ES/BS (%)	9	7.939	5.114	10.764	7.386	2.215	15.493	13.507	3.675	1.225	0.774	1.964
Osteoclast surface Oc.S/BS (%)	9	1.665	0.957	2.374	1.475	0.316	2.895	0.849	0.922	0.307	0.075	-1.397
Osteoclast number N.Oc/T.A. (/mm <sup>2</sup> )	9	0.158	0.092	0.224	0.136	0.037	0.295	0.007	0.086	0.029	0.279	-1.076
Mineralising surface MS/BS (%)	9	9.288	7.367	11.210	9.434	5.495	13.667	6.248	2.500	0.833	0.231	0.072
Osteoid apposition rate OAR xi	9	0.961	0.799	1.124	0.975	0.665	1.268	0.045	0.212	0.071	-0.049	-1.324
Mineralisation lag time Mlt (days)	9	0.323	0.223	0.422	0.372	0.096	0.459	0.017	0.129	0.043	-0.696	-0.837
Bone formation rate BFR/BS (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	9	33.369	23.063	43.675	32.429	13.332	57.037	179.765	13.408	4.469	0.334	-0.204
Rel mineral Vol	9	97.676	96.729	98.623	98.069	95.122	99.164	1.518	1.232	0.411	-1.134	1.206
Surface Density	9	2.901	2.365	3.437	2.635	2.186	4.544	0.486	0.697	0.232	1.925	4.109
Resting Surface	9	80.525	75.661	85.390	81.121	70.411	89.873	40.054	6.329	2.110	-0.066	-0.841
Surf dens ost seams	9	0.314	0.237	0.391	0.298	0.203	0.478	0.010	0.100	0.033	0.563	-1.142
Surf dens ostoid osteoblast interface	9	0.024	0.008	0.041	0.031	0.000	0.064	0.000	0.021	0.007	0.491	-0.059
Ostoid thickness index	9	20.278	15.804	24.752	22.341	13.490	28.499	33.879	5.821	1.940	-0.046	-1.849
Surface density of Howship's lacunae	9	0.247	0.105	0.389	0.208	0.057	0.704	0.034	0.185	0.062	2.207	5.854
Surface density of bone osteoclast interface	9	0.046	0.029	0.064	0.049	0.008	0.076	0.001	0.023	0.008	-0.377	-0.645
Total osteoclasts (v)	9	0.441	0.273	0.608	0.435	0.094	0.832	0.047	0.218	0.073	0.325	0.289
Bone osteoclasts (TRS)	9	2.132	1.353	2.911	2.063	0.656	3.999	1.028	1.014	0.338	0.410	0.274
Fractional labeled surfaces	9	13.611	10.530	16.693	13.962	8.791	22.000	16.074	4.009	1.336	0.933	1.670
Fractional double labeled surfaces	9	4.965	3.426	6.504	4.906	2.198	8.155	4.009	2.002	0.667	0.194	-0.480



Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: *Descriptive statistics*

#### Descriptive Statistics

OVX-St	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Std.Dev.	Standard		
			-95.000%	+95.000%						Error	Skewness	Kurtosis
Bone Volume (BV/TV)(%)	10	9.533	6.882	12.185	7.880	6.801	18.989	13.734	3.706	1.172	2.197	5.131
Osteoid volume OV/BV (%)	10	2.632	1.334	3.931	1.971	0.480	6.280	3.295	1.815	0.574	0.905	0.175
Osteoid volume OV/TV (%)	10	0.225	0.127	0.322	0.204	0.074	0.481	0.019	0.136	0.043	0.679	-0.480
Osteoid Surface OS/BS (%)	10	13.594	9.281	17.907	12.178	6.630	23.209	36.350	6.029	1.907	0.601	-1.229
Osteoblast surface Ob.S/BS (%)	10	1.376	0.496	2.257	0.959	0.000	3.659	1.515	1.231	0.389	1.220	0.422
Osteoid thickness O.Th (mcm)	10	8.149	5.676	10.622	7.680	2.566	14.258	11.953	3.457	1.093	0.662	0.686
Eroded surface ES/BS (%)	10	8.145	6.279	10.012	7.686	4.673	13.189	6.807	2.609	0.825	0.515	0.053
Osteoclast surface Oc.S/BS (%)	10	1.694	1.121	2.267	1.649	0.287	2.744	0.641	0.801	0.253	-0.388	-0.660
Osteoclast number N.Oc/T.A. (/mm <sup>2</sup> )	10	0.158	0.110	0.206	0.181	0.065	0.247	0.005	0.068	0.021	-0.359	-1.504
Mineralising surface MS/BS (%)	10	8.837	6.549	11.126	8.616	4.893	15.447	10.232	3.199	1.012	0.816	0.542
Osteoid apposition rate OAR xi	10	0.996	0.898	1.094	1.047	0.764	1.186	0.019	0.137	0.043	-0.553	-0.647
Mineralisation lag time Mlt (days)	10	0.274	0.187	0.361	0.285	0.122	0.460	0.015	0.122	0.038	0.105	-1.635
Bone formation rate BFR/BS (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	10	31.933	24.005	39.861	33.342	17.742	45.477	122.818	11.082	3.505	-0.106	-2.015
Rel mineral Vol	10	97.368	96.069	98.666	98.029	93.720	99.520	3.295	1.815	0.574	-0.905	0.175
Surface Density	10	2.685	2.023	3.346	2.395	1.961	5.106	0.854	0.924	0.292	2.324	6.136
Resting Surface	10	78.261	73.685	82.837	79.122	67.335	88.398	40.926	6.397	2.023	-0.361	-0.359
Surf dens ost seams	10	0.354	0.239	0.470	0.344	0.149	0.691	0.026	0.162	0.051	0.720	0.888
Surf dens ostoid osteoblast interface	10	0.037	0.013	0.061	0.022	0.000	0.102	0.001	0.033	0.011	1.042	-0.049
Ostoid thickness index	10	18.043	13.170	22.915	17.146	5.421	31.097	46.394	6.811	2.154	0.239	1.548
Surface density of Howship's lacunae	10	0.232	0.114	0.351	0.162	0.125	0.673	0.027	0.166	0.052	2.504	6.742
Surface density of bone osteoclast interface	10	0.045	0.024	0.067	0.036	0.009	0.119	0.001	0.030	0.010	1.733	4.250
Total osteoclasts (v)	10	0.421	0.234	0.607	0.389	0.180	1.083	0.068	0.261	0.082	2.023	5.119
Bone osteoclasts (TRS)	10	2.078	1.349	2.806	2.044	0.700	3.851	1.036	1.018	0.322	0.335	-0.570
Fractional labeled surfaces	10	13.077	9.784	16.371	13.407	6.422	21.951	21.193	4.604	1.456	0.466	0.097
Fractional double labeled surfaces	10	4.597	3.090	6.104	4.207	2.326	8.943	4.436	2.106	0.666	1.106	0.721

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH:. Statistical analyses - Mann Whitney U-test

**Mann-Whitney U Test**

By variable: GROUPS

Group 1: 102-OVX Group 2: 100-Sh

	Rank Sum		U	Z	p-level	Z		Valid N	Valid N	2*1sided exact p
	OVX	Sham				adjusted	p-level			
Bone Volume (BV/TV)(%)	48	123	3	-3.3113	0.0009	-3.3113	0.0009	9	9	0.0003
Osteoid volume OV/BV (%)	119	52	7	2.9581	0.0031	2.9581	0.0031	9	9	0.0019
Osteoid volume OV/TV (%)	111	60	15	2.2517	0.0243	2.2575	0.0240	9	9	0.0244
Osteoid Surface OS/BS (%)	120	51	6	3.0464	0.0023	3.0464	0.0023	9	9	0.0012
Osteoblast surface Ob.S/BS (%)	97	74	29	1.0155	0.3099	1.0160	0.3096	9	9	0.3401
Osteoid thickness O.Th (mcm)	97.5	73.5	28.5	1.0596	0.2893	1.0602	0.2891	9	9	0.2973
Eroded surface ES/BS (%)	100	71	26	1.2804	0.2004	1.2804	0.2004	9	9	0.2224
Osteoclast surface Oc.S/BS (%)	110	61	16	2.1634	0.0305	2.1634	0.0305	9	9	0.0315
Osteoclast number N.Oc/T.A. (/mm <sup>2</sup> )	115	56	11	2.6049	0.0092	2.6063	0.0092	9	9	0.0078
Mineralising surface MS/BS (%)	117	54	9	2.7815	0.0054	2.7815	0.0054	9	9	0.0040
Osteoid apposition rate OAR xi	101	70	25	1.3687	0.1711	1.3687	0.1711	9	9	0.1903
Mineralisation lag time Mlt (days)	60	111	15	-2.2517	0.0243	-2.2517	0.0243	9	9	0.0244
Bone formation rate BFR/BS (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	117	54	9	2.7815	0.0054	2.7815	0.0054	9	9	0.0040
Rel mineral Vol	52	119	7	-2.9581	0.0031	-2.9581	0.0031	9	9	0.0019
Surface Density	48	123	3	-3.3113	0.0009	-3.3113	0.0009	9	9	0.0003
Resting Surface	53	118	8	-2.8698	0.0041	-2.8698	0.0041	9	9	0.0028
Surf dens ost seams	108	63	18	1.9868	0.0470	1.9868	0.0470	9	9	0.0503
Surf dens ostoid osteoblast interface	83.5	87.5	38.5	-0.1766	0.8598	-0.1769	0.8596	9	9	0.8633
Ostoid thickness index	101	70	25	1.3687	0.1711	1.3687	0.1711	9	9	0.1903
Surface density of Howship's lacunae	69	102	24	-1.4570	0.1451	-1.4577	0.1449	9	9	0.1615
Surface density of bone osteoclast interface	96.5	74.5	29.5	0.9713	0.3314	0.9723	0.3309	9	9	0.3401
Total osteoclasts (v)	104	67	22	1.6336	0.1024	1.6361	0.1018	9	9	0.1135
Bone osteoclasts (TRS)	114	57	12	2.5166	0.0119	2.5166	0.0119	9	9	0.0106
Fractional labeled surfaces	110	61	16	2.1634	0.0305	2.1634	0.0305	9	9	0.0315
Fractional double labeled surfaces	123	48	3	3.3113	0.0009	3.3113	0.0009	9	9	0.0003

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: *Statistical analyses - Mann Whitney U-test*

Mann-Whitney U Test

By variable GROUPS

Group 1: 100-Sham Group 2: 101-Sham Sta

	Rank Sum		U	Z	p-level	Z		Valid N Sham	Valid N Sham Sta	2*1sided exact p
	Sham	Sham Sta				adjusted	p-level			
Bone Volume (BV/TV)(%)	93	97	42	0.2449	0.8065	0.2449	0.8065	9	10	0.8421
Osteoid volume OV/BV (%)	63	127	18	-2.2045	0.0275	-2.2045	0.0275	9	10	0.0279
Osteoid volume OV/TV (%)	62.5	127.5	17.5	-2.2454	0.0248	-2.2503	0.0244	9	10	0.0220
Osteoid Surface OS/BS (%)	60	130	15	-2.4495	0.0143	-2.4495	0.0143	9	10	0.0133
Osteoblast surface Ob.S/BS (%)	60	130	15	-2.4495	0.0143	-2.4495	0.0143	9	10	0.0133
Osteoid thickness O.Th (mcm)	96.5	93.5	38.5	0.5307	0.5956	0.5310	0.5955	9	10	0.6038
Eroded surface ES/BS (%)	65	125	20	-2.0412	0.0412	-2.0412	0.0412	9	10	0.0435
Osteoclast surface Oc.S/BS (%)	65	125	20	-2.0412	0.0412	-2.0412	0.0412	9	10	0.0435
Osteoclast number N.Oc/T.A. (/mm <sup>2</sup> )	55	135	10	-2.8577	0.0043	-2.8590	0.0043	9	10	0.0030
Mineralising surface MS/BS (%)	89	101	44	-0.0816	0.9349	-0.0816	0.9349	9	10	0.9682
Osteoid apposition rate OAR xi	89	101	44	-0.0816	0.9349	-0.0816	0.9349	9	10	0.9682
Mineralisation lag time Mlt (days)	92	98	43	0.1633	0.8703	0.1633	0.8703	9	10	0.9048
Bone formation rate BFR/BS (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	86	104	41	-0.3266	0.7440	-0.3266	0.7440	9	10	0.7802
Rel mineral Vol	117	73	18	2.2045	0.0275	2.2045	0.0275	9	10	0.0279
Surface Density	97	93	38	0.5715	0.5676	0.5715	0.5676	9	10	0.6038
Resting Surface	124	66	11	2.7761	0.0055	2.7761	0.0055	9	10	0.0041
Surf dens ost seams	58	132	13	-2.6128	0.0090	-2.6128	0.0090	9	10	0.0076
Surf dens ostoid osteoblast interface	62	128	17	-2.2862	0.0222	-2.2912	0.0220	9	10	0.0220
Ostoid thickness index	96	94	39	0.4899	0.6242	0.4899	0.6242	9	10	0.6607
Surface density of Howship's lacunae	71	119	26	-1.5513	0.1208	-1.5520	0.1207	9	10	0.1333
Surface density of bone osteoclast interface	68.5	121.5	23.5	-1.7555	0.0792	-1.7570	0.0789	9	10	0.0789
Total osteoclasts (v)	60	130	15	-2.4495	0.0143	-2.4527	0.0142	9	10	0.0133
Bone osteoclasts (TRS)	70	120	25	-1.6330	0.1025	-1.6330	0.1025	9	10	0.1128
Fractional labeled surfaces	95	95	40	0.4082	0.6831	0.4082	0.6831	9	10	0.7197
Fractional double labeled surfaces	70	120	25	-1.6330	0.1025	-1.6330	0.1025	9	10	0.1128

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: Statistical analyses - Mann Whitney U-test

Mann-Whitney U Test (st1 histo data corelation.sta)

By variable GROUPS

Group 1: 102-OVX Group 2: 103-OVX Stat

	Rank Sum OVX	Rank Sum OVX Stat	U	Z	p-level	Z adjusted	p-level	Valid N OVX	Valid N OVX Stat	2*1sided exact p
Bone Volume (BV/TV)(%)	110	80	25	1.6330	0.1025	1.6330	0.1025	9	10	0.1128
Osteoid volume OV/BV (%)	88	102	43	-0.1633	0.8703	-0.1633	0.8703	9	10	0.9048
Osteoid volume OV/TV (%)	92	98	43	0.1633	0.8703	0.1635	0.8701	9	10	0.9048
Osteoid Surface OS/BS (%)	78	112	33	-0.9798	0.3272	-0.9798	0.3272	9	10	0.3562
Osteoblast surface Ob.S/BS (%)	81	109	36	-0.7348	0.4624	-0.7361	0.4617	9	10	0.4967
Osteoid thickness O.Th (mcm)	96	94	39	0.4899	0.6242	0.4903	0.6239	9	10	0.6607
Eroded surface ES/BS (%)	90	100	45	0.0000	1.0000	0.0000	1.0000	9	10	1.0318
Osteoclast surface Oc.S/BS (%)	89	101	44	-0.0816	0.9349	-0.0816	0.9349	9	10	0.9682
Osteoclast number N.Oc/T.A. (/mm2)	93	97	42	0.2449	0.8065	0.2449	0.8065	9	10	0.8421
Mineralising surface MS/BS (%)	96	94	39	0.4899	0.6242	0.4899	0.6242	9	10	0.6607
Osteoid apposition rate OAR xi	84	106	39	-0.4899	0.6242	-0.4901	0.6241	9	10	0.6607
Mineralisation lag time Mlt (days)	101	89	34	0.8981	0.3691	0.8981	0.3691	9	10	0.4002
Bone formation rate BFR/BS (mcm3/mcm2/yr)	92	98	43	0.1633	0.8703	0.1633	0.8703	9	10	0.9048
Rel mineral Vol	92	98	43	0.1633	0.8703	0.1633	0.8703	9	10	0.9048
Surface Density	104	86	31	1.1431	0.2530	1.1431	0.2530	9	10	0.2775
Resting Surface	97	93	38	0.5715	0.5676	0.5715	0.5676	9	10	0.6038
Surf dens ost seams	82.5	107.5	37.5	-0.6124	0.5403	-0.6126	0.5401	9	10	0.5490
Surf dens ostoid osteoblast interface	82.5	107.5	37.5	-0.6124	0.5403	-0.6137	0.5394	9	10	0.5490
Ostoid thickness index	96	94	39	0.4899	0.6242	0.4899	0.6242	9	10	0.6607
Surface density of Howship's lacunae	93.5	96.5	41.5	0.2858	0.7751	0.2859	0.7750	9	10	0.7802
Surface density of bone osteoclast interface	100.5	89.5	34.5	0.8573	0.3913	0.8581	0.3909	9	10	0.4002
Total osteoclasts (v)	96	94	39	0.4899	0.6242	0.4901	0.6241	9	10	0.6607
Bone osteoclasts (TRS)	92	98	43	0.1633	0.8703	0.1633	0.8703	9	10	0.9048
Fractional labeled surfaces	93	97	42	0.2449	0.8065	0.2449	0.8065	9	10	0.8421
Fractional double labeled surfaces	97	93	38	0.5715	0.5676	0.5715	0.5676	9	10	0.6038

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: Statistical analyses - ANOVA; All effects

GROUPS; LS Means

Wilks lambda=.09727, F(33, 71.412)=2.6086, **p=-.00037**

#### Effective hypothesis decomposition

	BONE VOL	BONE VOL	BONE VOL	BONE VOL	OST VOL	OST VOL	OST VOL	OST VOL	OST VOL	OST VOL	OST VOL	OST VOL
GROUPS	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%
Sham	18.0152	1.1537	15.6705	20.3598	0.8014	0.4221	-0.0563	1.6591	0.1305	0.0440	0.0412	0.2198
Sham Sta	17.2859	1.0945	15.0615	19.5102	1.5537	0.4004	0.7400	2.3674	0.2646	0.0417	0.1798	0.3493
OVX	10.5402	1.1537	8.1955	12.8849	2.3244	0.4221	1.4667	3.1821	0.2289	0.0440	0.1396	0.3182
OVX Stat	9.5335	1.0945	7.3091	11.7578	2.6325	0.4004	1.8188	3.4462	0.2245	0.0417	0.1398	0.3093
	OST SURF	OST SURF	OST SURF	OST SURF	OB SURF	OB SURF	OB SURF	OB SURF	EROD SURF	EROD SURF	EROD SURF	EROD SURF
GROUPS	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%
Sham	4.4079	1.6126	1.1307	7.6851	0.4917	0.2529	-0.0222	1.0056	6.0550	0.9485	4.1274	7.9826
Sham Sta	9.5337	1.5299	6.4247	12.6428	1.1168	0.2399	0.6293	1.6043	8.1096	0.8998	6.2809	9.9383
OVX	11.5353	1.6126	8.2580	14.8125	0.7730	0.2529	0.2591	1.2869	7.9395	0.9485	6.0119	9.8671
OVX Stat	13.5938	1.5299	10.4848	16.7029	1.3765	0.2399	0.8890	1.8640	8.1451	0.8998	6.3164	9.9738
	OC SURF	OC SURF	OC SURF	OC SURF	OC NUMB	OC NUMB	OC NUMB	OC NUMB	BFR	BFR	BFR	BFR
GROUPS	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%
Sham	0.7361	0.2239	0.2811	1.1912	0.0570	0.0196	0.0171	0.0968	15.4496	3.3547	8.6321	22.2671
Sham Sta	1.2051	0.2124	0.7734	1.6368	0.1092	0.0186	0.0714	0.1470	15.6503	3.1825	9.1826	22.1180
OVX	1.6651	0.2239	1.2100	2.1202	0.1584	0.0196	0.1186	0.1983	33.3692	3.3547	26.5516	40.1867
OVX Stat	1.6937	0.2124	1.2620	2.1254	0.1581	0.0186	0.1203	0.1959	31.9327	3.1825	25.4651	38.4004
	TOT OC	TOT OC	TOT OC	TOT OC	X_BONE_O	BONE OC	BONE OC	BONE OC				
GROUPS	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%				
Sham	0.3018	0.0766	0.1462	0.4574	0.9721	0.2702	0.4231	1.5212				
Sham Sta	0.5486	0.0726	0.4010	0.6962	1.4763	0.2563	0.9553	1.9972				
OVX	0.4407	0.0766	0.2851	0.5963	2.1320	0.2702	1.5829	2.6811				
OVX Stat	0.4205	0.0726	0.2729	0.5681	2.0777	0.2563	1.5568	2.5986				

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: *Statistical analyses - ANOVA; Between group analysis*

#### LSD test; variable BONE VOL (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = 11.980, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	18.015	17.286	10.540	9.5335
1	<b>Sham</b>		0.64945314	5.95564E-05	6.34029E-06
2	<b>Sham Sta</b>	0.64945314		0.000161122	1.67577E-05
3	<b>OVX</b>	5.9556E-05	0.000161122		0.530959101
4	<b>OVX Stat</b>	6.3403E-06	1.67577E-05	0.530959101	

#### LSD test; variable OST VOL (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = 1.6031, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	.80144	1.5537	2.3244	2.6325
1	<b>Sham</b>		0.204680899	0.015392146	0.003418107
2	<b>Sham Sta</b>	0.2046809		0.19411176	0.06524982
3	<b>OVX</b>	0.01539215	0.19411176		0.599807789
4	<b>OVX Stat</b>	0.00341811	0.06524982	0.599807789	

#### LSD test; variable OST VOL (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .01739, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	.13049	.26455	.22892	.22452
1	<b>Sham</b>		0.033728343	0.12256407	0.129933252
2	<b>Sham Sta</b>	0.03372834		0.560322421	0.501767734
3	<b>OVX</b>	0.12256407	0.560322421		0.942475691
4	<b>OVX Stat</b>	0.12993325	0.501767734	0.942475691	

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: Statistical analyses - ANOVA; Between group analysis

#### LSD test; variable OST SURF (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = 23.405, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	4.4079	9.5337	11.535	13.594
1	<b>Sham</b>		0.027337866	0.003624295	0.00022112
2	<b>Sham Sta</b>	0.02733787		0.374222464	0.069179039
3	<b>OVX</b>	0.00362429	0.374222464		0.36092041
4	<b>OVX Stat</b>	0.00022112	0.069179039	0.36092041	

#### LSD test; variable E\_OSTEOB (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .57547, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	.49171	1.1168	.77302	1.3765
1	<b>Sham</b>		0.08181064	0.436940221	0.015881465
2	<b>Sham Sta</b>	0.08181064		0.330949266	0.449254162
3	<b>OVX</b>	0.43694022	0.330949266		0.092447917
4	<b>OVX Stat</b>	0.01588146	0.449254162	0.092447917	

#### LSD test; variable G\_ERODED (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = 8.0971, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	6.0550	8.1096	7.9395	8.1451
1	<b>Sham</b>		0.125328797	0.169132377	0.1191582
2	<b>Sham Sta</b>	0.1253288		0.897228138	0.97792925
3	<b>OVX</b>	0.16913238	0.897228138		0.875970902
4	<b>OVX Stat</b>	0.1191582	0.97792925	0.875970902	

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: Statistical analyses - ANOVA; Between group analysis

#### LSD test; variable H\_OSTEOC (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .45127, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	.73614	1.2051	1.6651	1.6937
1	<b>Sham</b>		0.13788824	0.005963527	0.003848783
2	<b>Sham Sta</b>	0.13788824		0.145381262	0.113127307
3	<b>OVX</b>	0.00596353	0.145381262		0.926721783
4	<b>OVX Stat</b>	0.00384878	0.113127307	0.926721783	

#### LSD test; variable J\_OSTEOC (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .00346, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	.05695	.10919	.15842	.15807
1	<b>Sham</b>		0.061572805	0.000847199	0.000673397
2	<b>Sham Sta</b>	0.06157281		0.077239163	0.071762864
3	<b>OVX</b>	0.0008472	0.077239163		0.989588355
4	<b>OVX Stat</b>	0.0006734	0.071762864	0.989588355	

#### LSD test; variable N\_BONE\_F (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = 101.28, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	15.450	15.650	33.369	31.933
1	<b>Sham</b>		0.965635799	0.000610337	0.001105271
2	<b>Sham Sta</b>	0.9656358		0.000522886	0.000953901
3	<b>OVX</b>	0.00061034	0.000522886		0.757969564
4	<b>OVX Stat</b>	0.00110527	0.000953901	0.757969564	



Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: QBH: *Statistical analyses - ANOVA; Between group analysis*

**LSD test; variable W\_TOTAL (anova histo.sta)**

Probabilities for Post Hoc Tests

Error: Between MS = .05275, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	.30183	.54864	.44073	.42055
1	<b>Sham</b>		0.02536602	0.20819934	0.268489264
2	<b>Sham Sta</b>	0.02536602		0.313771367	0.220906142
3	<b>OVX</b>	0.20819934	0.313771367		0.849435496
4	<b>OVX Stat</b>	0.26848926	0.220906142	0.849435496	

**LSD test; variable X\_BONE\_O (anova histo.sta)**

Probabilities for Post Hoc Tests

Error: Between MS = .65701, df = 34.000

		{1}	{2}	{3}	{4}
	<b>GROUPS</b>	.97214	1.4763	2.1320	2.0777
1	<b>Sham</b>		0.184797994	0.004583901	0.005451994
2	<b>Sham Sta</b>	0.18479799		0.087279328	0.106296542
3	<b>OVX</b>	0.0045839	0.087279328		0.884875618
4	<b>OVX Stat</b>	0.00545199	0.106296542	0.884875618	

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: BMD delta values. *Descriptive statistics*

#### Descriptive Statistics (bmd 1 and 2 femur.sta)

	Valid N	Mean	Confid. -95.000%	Confid. +95.000%	Median	Minimum	Maximum	Variance	Std.Dev.	Standard Error	Skewness	Kurtosis
SH_DELTA	10	-0.00432	-0.00668	-0.00196	-0.0041	-0.0098	0.0008	1.09E-05	0.003304	0.001045	-0.33443	-0.25984
OVX_DELT	10	-6E-05	-0.00133	0.001215	-0.00065	-0.0019	0.0032	3.18E-06	0.001782	0.000564	0.919437	-0.37823

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: BMD delta values. *Statistical analyses - ANOVA; All effects*

#### GROUP; LS Means (bmd 1 and 2 femur.sta)

Current effect: F(1, 18)=12.879, p=.00210

Effective hypothesis decomposition

	GROUP	DELTAS Mean	DELTAS Std.Err.	DELTAS -95.00%	DELTAS +95.00%	N
1	Sh	-0.00432	0.000839	-0.00608	-0.00256	10
2	OVX	-6E-05	0.000839	-0.00182	0.001703	10

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: BMD delta values. *Statistical analyses - ANOVA; Between group analysis*

#### LSD test; variable DELTAS (bmd 1 and 2 femur.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .00001, df = 18.000

	GROUP	{1}	{2}
1	Sh	-.0043	-.0001
2	OVX	0.002099	0.002099

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Rat Weights. *Descriptive statistics.*

**Descriptive Statistics: Sh weights**

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Range	Variance	Std.Dev.	Standard	
			-95.000%	+95.000%							Error	
<b>W1</b>	10	246.600	233.789	259.411	248.500	223.000	274.000	51.000	320.711	17.908	5.663	
<b>W2</b>	10	251.100	235.484	266.716	250.000	221.000	297.000	76.000	476.544	21.830	6.903	
<b>W3</b>	10	245.900	232.313	259.487	249.500	215.000	274.000	59.000	360.767	18.994	6.006	
<b>W4</b>	10	233.400	220.435	246.365	233.500	207.000	265.000	58.000	328.489	18.124	5.731	
<b>W5</b>	10	234.700	221.458	247.942	239.500	204.000	261.000	57.000	342.678	18.512	5.854	
<b>W6</b>	10	231.700	218.670	244.730	230.500	206.000	264.000	58.000	331.789	18.215	5.760	
<b>W7</b>	10	249.600	237.520	261.680	251.000	219.000	279.000	60.000	285.156	16.887	5.340	
<b>W8</b>	10	261.600	249.662	273.538	260.000	236.000	289.000	53.000	278.489	16.688	5.277	

**Descriptive Statistics: OVX weights**

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Range	Variance	Std.Dev.	Standard	
			-95.000%	+95.000%							Error	
<b>W1</b>	10	247.000	235.684	258.316	241.000	229.000	271.000	42.000	250.222	15.818	5.002	
<b>W2</b>	10	265.100	253.338	276.862	263.500	243.000	290.000	47.000	270.322	16.441	5.199	
<b>W3</b>	10	256.900	245.919	267.881	260.000	231.000	277.000	46.000	235.656	15.351	4.854	
<b>W4</b>	10	245.000	234.151	255.849	247.000	220.000	267.000	47.000	230.000	15.166	4.796	
<b>W5</b>	10	244.600	234.075	255.125	245.000	220.000	266.000	46.000	216.489	14.714	4.653	
<b>W6</b>	10	247.900	241.152	254.648	249.500	230.000	266.000	36.000	88.989	9.433	2.983	
<b>W7</b>	10	262.000	252.691	271.309	263.000	242.000	287.000	45.000	169.333	13.013	4.115	
<b>W8</b>	10	278.500	267.535	289.465	278.000	256.000	308.000	52.000	234.944	15.328	4.847	

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Rat Weights. *Descriptive statistics.*

#### Descriptive Statistics: Sh-St weights.

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Range	Variance	Std.Dev.	Standard
			-95.000%	+95.000%							Error
<b>W1</b>	10	236.100	221.559	250.641	228.000	216.000	263.000	47.000	413.211	20.328	6.428
<b>W2</b>	10	239.000	225.381	252.619	232.000	219.000	264.000	45.000	362.444	19.038	6.020
<b>W3</b>	10	237.100	223.019	251.181	230.000	216.000	262.000	46.000	387.433	19.683	6.224
<b>W4</b>	10	223.500	209.772	237.228	218.500	199.000	252.000	53.000	368.278	19.191	6.069
<b>W5</b>	10	222.200	209.949	234.451	219.500	199.000	244.000	45.000	293.289	17.126	5.416
<b>W6</b>	10	222.300	210.301	234.299	220.500	203.000	245.000	42.000	281.344	16.773	5.304
<b>W7</b>	10	237.400	226.831	247.969	234.500	216.000	257.000	41.000	218.267	14.774	4.672
<b>W8</b>	10	254.600	241.387	267.813	249.000	234.000	280.000	46.000	341.156	18.470	5.841

#### Descriptive Statistics: OVX-St weights.

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Range	Variance	Std.Dev.	Standard
			-95.000%	+95.000%							Error
<b>W1</b>	10	255.200	241.582	268.818	255.500	229.000	285.000	56.000	362.400	19.037	6.020
<b>W2</b>	10	269.700	256.744	282.656	270.500	242.000	299.000	57.000	328.011	18.111	5.727
<b>W3</b>	10	266.500	252.755	280.245	265.500	236.000	296.000	60.000	369.167	19.214	6.076
<b>W4</b>	10	252.900	239.084	266.716	249.500	222.000	290.000	68.000	372.989	19.313	6.107
<b>W5</b>	10	250.500	234.485	266.515	241.500	219.000	290.000	71.000	501.167	22.387	7.079
<b>W6</b>	10	248.100	234.301	261.899	240.500	221.000	283.000	62.000	372.100	19.290	6.100
<b>W7</b>	10	263.500	248.094	278.906	257.000	228.000	301.000	73.000	463.833	21.537	6.811
<b>W8</b>	10	279.000	263.896	294.104	280.000	238.000	310.000	72.000	445.778	21.113	6.677

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Rat Weights. *Statistical analyses - Mann Whitney U-test.*

Mann-Whitney U Test (statbones 1 weights1.sta)  
By variable GROUP

**Group 1: 100-Sh Group 2: 101-Sh-S**

	Rank Sum		U	Z	p-level	adjusted	p-level	Valid N		2*1sided exact p
	Sh	Sh-S						Sh	Sh-S	
<b>W1</b>	121.5	88.5	33.500	1.247	0.212	1.250	0.211	10	10	0.218
<b>W2</b>	120.5	89.5	34.500	1.172	0.241	1.173	0.241	10	10	0.247
<b>W3</b>	114.0	96.0	41.000	0.680	0.496	0.682	0.495	10	10	0.529
<b>W4</b>	117.0	93.0	38.000	0.907	0.364	0.909	0.363	10	10	0.393
<b>W5</b>	125.5	84.5	29.500	1.550	0.121	1.551	0.121	10	10	0.123
<b>W6</b>	120.5	89.5	34.500	1.172	0.241	1.173	0.241	10	10	0.247
<b>W7</b>	124.0	86.0	31.000	1.436	0.151	1.437	0.151	10	10	0.165
<b>W8</b>	117.0	93.0	38.000	0.907	0.364	0.908	0.364	10	10	0.393

Mann-Whitney U Test (statbones 1 weights1.sta)  
By variable GROUP

**Group 1: 100-Sh Group 2: 102-OVX**

	Rank Sum		U	Z	p-level	adjusted	p-level	Valid N		2*1sided exact p
	Sh	OVX						Sh	OVX	
<b>W1</b>	104.5	105.5	49.500	-0.038	0.970	-0.038	0.970	10	10	0.971
<b>W2</b>	84.0	126.0	29.000	-1.587	0.112	-1.589	0.112	10	10	0.123
<b>W3</b>	87.5	122.5	32.500	-1.323	0.186	-1.323	0.186	10	10	0.190
<b>W4</b>	87.0	123.0	32.000	-1.361	0.174	-1.362	0.173	10	10	0.190
<b>W5</b>	89.5	120.5	34.500	-1.172	0.241	-1.173	0.241	10	10	0.247
<b>W6</b>	74.0	136.0	19.000	-2.343	0.019	-2.344	0.019	10	10	0.019
<b>W7</b>	84.0	126.0	29.000	-1.587	0.112	-1.589	0.112	10	10	0.123
<b>W8</b>	78.0	132.0	23.000	-2.041	0.041	-2.043	0.041	10	10	0.043

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Rat Weights. *Statistical analyses - Mann Whitney U-test.*

Mann-Whitney U Test (statbones 1 weights1.sta)

By variable GROUP

Group 1: 100-Sh Group 2: 103-OVX-S

	Rank Sum		U	Z	p-level	Z		Valid N		2*1sided exact p
	Sh	OVX-S				adjusted	p-level	Sh	OVX-S	
<b>W1</b>	91.0	119.0	36.000	-1.058	0.290	-1.060	0.289	10	10	0.315
<b>W2</b>	79.5	130.5	24.500	-1.928	0.054	-1.928	0.054	10	10	0.052
<b>W3</b>	77.0	133.0	22.000	-2.117	0.034	-2.117	0.034	10	10	0.035
<b>W4</b>	78.0	132.0	23.000	-2.041	0.041	-2.042	0.041	10	10	0.043
<b>W5</b>	88.5	121.5	33.500	-1.247	0.212	-1.250	0.211	10	10	0.218
<b>W6</b>	85.0	125.0	30.000	-1.512	0.131	-1.514	0.130	10	10	0.143
<b>W7</b>	83.5	126.5	28.500	-1.625	0.104	-1.628	0.103	10	10	0.105
<b>W8</b>	81.0	129.0	26.000	-1.814	0.070	-1.816	0.069	10	10	0.075

Mann-Whitney U Test (statbones 1 weights1.sta)

By variable GROUP

Group 1: 101-Sh-S Group 2: 102-OVX

	Rank Sum		U	Z	p-level	Z		Valid N		2*1sided exact p
	Sh-S	OVX				adjusted	p-level	Sh-S	OVX	
<b>W1</b>	85.5	124.5	30.500	-1.474	0.140	-1.476	0.140	10	10	0.143
<b>W2</b>	71.0	139.0	16.000	-2.570	0.010	-2.573	0.010	10	10	0.009
<b>W3</b>	75.0	135.0	20.000	-2.268	0.023	-2.269	0.023	10	10	0.023
<b>W4</b>	73.5	136.5	18.500	-2.381	0.017	-2.383	0.017	10	10	0.015
<b>W5</b>	70.0	140.0	15.000	-2.646	0.008	-2.648	0.008	10	10	0.007
<b>W6</b>	63.5	146.5	8.500	-3.137	0.002	-3.139	0.002	10	10	0.001
<b>W7</b>	65.0	145.0	10.000	-3.024	0.002	-3.024	0.002	10	10	0.002
<b>W8</b>	71.5	138.5	16.500	-2.532	0.011	-2.534	0.011	10	10	0.009

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Rat Weights. *Statistical analyses - Mann Whitney U-test.*

Mann-Whitney U Test (statbones 1 weights1.sta)

By variable GROUP

Group 1: 101-Sh-S Group 2: 103-OVX-S

	Rank Sum		U	Z	p-level	Z		Valid N		2*1sided exact p
	Sh-S	OVX-S				adjusted	p-level	Sh-S	OVX-S	
W1	77.0	133.0	22.000	-2.117	0.034	-2.121	0.034	10	10	0.035
W2	68.5	141.5	13.500	-2.759	0.006	-2.762	0.006	10	10	0.004
W3	70.0	140.0	15.000	-2.646	0.008	-2.648	0.008	10	10	0.007
W4	69.5	140.5	14.500	-2.684	0.007	-2.686	0.007	10	10	0.005
W5	75.0	135.0	20.000	-2.268	0.023	-2.269	0.023	10	10	0.023
W6	75.5	134.5	20.500	-2.230	0.026	-2.233	0.026	10	10	0.023
W7	71.5	138.5	16.500	-2.532	0.011	-2.537	0.011	10	10	0.009
W8	73.5	136.5	18.500	-2.381	0.017	-2.387	0.017	10	10	0.015

Mann-Whitney U Test (statbones 1 weights1.sta)

By variable GROUP

Group 1: 102-OVX Group 2: 103-OVX-S

	Rank Sum		U	Z	p-level	Z		Valid N		2*1sided exact p
	OVX	OVX-S				adjusted	p-level	OVX	OVX-S	
W1	95.0	115.0	40.000	-0.756	0.450	-0.759	0.448	10	10	0.481
W2	98.0	112.0	43.000	-0.529	0.597	-0.530	0.596	10	10	0.631
W3	91.5	118.5	36.500	-1.021	0.307	-1.022	0.307	10	10	0.315
W4	97.0	113.0	42.000	-0.605	0.545	-0.605	0.545	10	10	0.579
W5	101.5	108.5	46.500	-0.265	0.791	-0.265	0.791	10	10	0.796
W6	116.0	94.0	39.000	0.832	0.406	0.833	0.405	10	10	0.436
W7	105.5	104.5	49.500	0.038	0.970	0.038	0.970	10	10	0.971
W8	101.0	109.0	46.000	-0.302	0.762	-0.302	0.762	10	10	0.796

Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Rat Weights. *Statistical analyses - Mann Whitney U-test.*

Mann-Whitney U Test (delta.sta)  
By variable GROUP

**Group 1: 100-Sham Group 2: 103-OVX-St**

	Rank Sum		U	Z	p-level	Z	p-level	Valid N		2*1sided exact p
	Sham	OVX-St						Sham	OVX-St	
DELTA	96	114	41	-0.680	0.496	-0.682	0.495	10	10	0.529

Mann-Whitney U Test (delta.sta)  
By variable GROUP

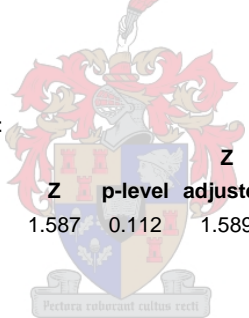
**Group 1: 101-OVX Group 2: 102-Sh-St**

	Rank Sum		U	Z	p-level	Z	p-level	Valid N		2*1sided exact p
	OVX	Sh-St						OVX	Sh-St	
DELTA	126	84	29	1.587	0.112	1.589	0.112	10	10	0.123

Mann-Whitney U Test (delta.sta)  
By variable GROUP

**Group 1: 100-Sham Group 2: 101-OVX**

	Rank Sum		U	Z	p-level	Z	p-level	Valid N		2*1sided exact p
	Sham	OVX						Sham	OVX	
DELTA	84.5	125.5	29.5	-1.550	0.121	-1.552	0.121	10	10	0.123





Study: 3.1. The effect of simvastatin 20mg/Kg/day administered for 8 weeks, on bone mineral density and quantitative bone histomorphometry, in sham-operated and ovariectomised female Sprague-Dawley rats.

### Study 3.1: Groups Sh, Sh-S, OVX, OVX-S: Rat Weights. *Statistical analyses - Mann Whitney U-test.*

Mann-Whitney U Test (delta.sta)

By variable GROUP

Group 1: 101-OVX Group 2: 103-OVX-St

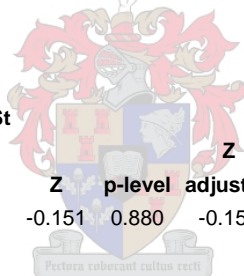
	Rank Sum		U	Z	p-level	adjusted	p-level	Valid N		2*1sided exact p
	OVX	OVX-St						OVX	OVX-St	
DELTA	116	94	39	0.832	0.406	0.833	0.405	10	10	0.436

Mann-Whitney U Test (delta.sta)

By variable GROUP

Group 1: 100-Sham Group 2: 102-Sh-St

	Rank Sum		U	Z	p-level	adjusted	p-level	Valid N		2*1sided exact p
	Sham	Sh-St						Sham	Sh-St	
DELTA	103	107	48	-0.151	0.880	-0.151	0.880	10	10	0.912



Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

## **Appendix B 3.2**

### **Study 3.2: Hard data, Descriptive statistics, Statistical analyses.**

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Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

### Study 3.2: Groups S20, C: Bone Mineral Density: Descriptive statistics

Descriptive Statistics (bmd 1 and 2 femur.sta)

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Std.Dev.	Standard		
			-95.000%	+95.000%						Error	Skewness	Kurtosis
<b>CONTROL</b>	10	0.1053	0.1012	0.1093	0.1039	0.0979	0.1161	0.0000	0.0057	0.0018	0.7839	-0.0845
<b>S20</b>	10	0.1031	0.0991	0.1071	0.1023	0.0941	0.1138	0.0000	0.0056	0.0018	0.5187	0.4303



Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

## Study 3.2: Groups S20, C: QBH: Descriptive statistics

Descriptive Statistics (data histo for graphs.sta)

S20	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Standard			
			-95.000%	+95.000%					Std.Dev.	Error	Skewness	Kurtosis
A Bone Volume (BV/TV)(%)	10	22.660	20.361	24.959	22.030	18.889	28.781	10.327	3.214	1.016	0.651	-0.479
B Osteoid volume OV/BV (%)	10	0.923	0.374	1.472	0.820	0.267	2.457	0.588	0.767	0.243	1.317	0.740
C Osteoid volume OV/TV (%)	10	0.202	0.093	0.312	0.187	0.051	0.483	0.024	0.154	0.049	1.013	-0.033
D Osteoid Surface OS/BS (%)	10	6.905	3.531	10.280	5.388	1.959	18.428	22.252	4.717	1.492	1.845	3.882
E Osteoblast surface Ob.S/BS (%)	10	1.755	0.797	2.712	1.486	0.309	4.072	1.792	1.339	0.423	1.079	0.067
F Osteoid thickness O.Th (mcm)	10	6.572	4.766	8.377	6.776	3.091	10.990	6.372	2.524	0.798	0.181	-0.528
G Eroded surface ES/BS (%)	10	9.351	7.851	10.850	9.361	5.740	12.263	4.394	2.096	0.663	-0.304	-0.849
H Osteoclast surface Oc.S/BS (%)	10	1.360	1.012	1.707	1.495	0.717	1.994	0.236	0.486	0.154	-0.060	-1.682
J Osteoclast number N.Oc/T.A. (/mm2)	10	0.114	0.084	0.145	0.118	0.052	0.173	0.002	0.043	0.014	-0.127	-1.426
K Mineralising surface MS/BS (%)	10	7.923	6.837	9.009	7.465	5.877	10.554	2.304	1.518	0.480	0.483	-0.961
L Osteoid apposition rate OAR xi	10	0.712	0.661	0.763	0.702	0.607	0.843	0.005	0.071	0.023	0.399	-0.347
M Mineralisation lag time Mlt (days)	10	0.334	0.230	0.438	0.332	0.139	0.526	0.021	0.146	0.046	-0.013	-1.888
N Bone formation rate BFR/BS (mcm3/mcm2/yr)	10	20.560	17.367	23.754	18.748	15.476	29.409	19.927	4.464	1.412	1.032	0.195
O Rel mineral Vol	10	99.077	98.528	99.626	99.180	97.543	99.733	0.588	0.767	0.243	-1.317	0.740
P Surface Density	10	5.612	5.032	6.192	5.461	4.586	7.244	0.658	0.811	0.257	0.790	0.310
Q Resting Surface	10	83.744	79.629	87.860	85.121	71.003	89.691	33.094	5.753	1.819	-1.342	1.654
R Surf dens ost seams	10	0.377	0.216	0.539	0.327	0.090	0.922	0.051	0.226	0.071	1.598	3.827
S Surf dens ostoid osteoblast interface	10	0.097	0.048	0.146	0.088	0.014	0.217	0.005	0.069	0.022	0.827	-0.350
T Ostoid thickness index	10	12.676	9.463	15.890	13.320	5.929	19.576	20.176	4.492	1.420	-0.116	-0.691
U Surface density of Howship's lacunae	10	0.525	0.415	0.636	0.534	0.333	0.888	0.024	0.154	0.049	1.395	3.089
V Surface density of bone osteoclast interface	10	0.077	0.053	0.101	0.078	0.038	0.144	0.001	0.034	0.011	0.714	0.184
W Total osteoclasts (v)	10	0.648	0.440	0.855	0.640	0.300	1.252	0.084	0.291	0.092	0.819	0.737
X Bone osteoclasts (TRS)	10	1.193	0.999	1.387	1.219	0.856	1.604	0.073	0.271	0.086	0.169	-1.496
Y Fractional labeled surfaces	10	13.194	11.438	14.951	12.623	9.634	17.647	6.030	2.456	0.777	0.391	-0.571
Z Fractional double labeled surfaces	10	2.652	2.062	3.243	2.297	1.765	4.035	0.681	0.826	0.261	0.875	-0.915

Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

## Study 3.2: Groups S20, C: QBH: Descriptive statistics

Descriptive Statistics (data histo for graphs.sta)

C	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Standard			
			-95.000%	+95.000%					Std.Dev.	Error	Skewness	Kurtosis
A Bone Volume (BV/TV)(%)	10	23.971	20.618	27.325	24.343	16.000	29.851	21.977	4.688	1.482	-0.447	-0.794
B Osteoid volume OV/BV (%)	10	0.557	0.426	0.688	0.649	0.240	0.714	0.033	0.183	0.058	-0.991	-0.764
C Osteoid volume OV/TV (%)	10	0.131	0.097	0.166	0.128	0.061	0.202	0.002	0.048	0.015	0.007	-0.681
D Osteoid Surface OS/BS (%)	10	4.657	3.729	5.585	4.684	2.582	7.285	1.682	1.297	0.410	0.610	1.110
E Osteoblast surface Ob.S/BS (%)	10	0.836	0.483	1.189	0.749	0.221	1.716	0.244	0.494	0.156	0.875	-0.079
F Osteoid thickness O.Th (mcm)	10	6.060	4.750	7.371	5.740	3.643	9.067	3.358	1.833	0.580	0.747	-0.330
G Eroded surface ES/BS (%)	10	7.388	6.617	8.160	7.334	5.978	9.379	1.164	1.079	0.341	0.471	-0.299
H Osteoclast surface Oc.S/BS (%)	10	0.799	0.646	0.952	0.816	0.516	1.188	0.046	0.213	0.068	0.414	-0.379
J Osteoclast number N.Oc/T.A. (/mm2)	10	0.065	0.052	0.079	0.068	0.041	0.098	0.000	0.019	0.006	0.198	-1.078
K Mineralising surface MS/BS (%)	10	7.053	5.921	8.185	7.296	4.421	8.811	2.503	1.582	0.500	-0.584	-1.021
L Osteoid apposition rate OAR xi	10	0.670	0.615	0.725	0.666	0.544	0.821	0.006	0.077	0.024	0.417	1.038
M Mineralisation lag time Mlt (days)	10	0.370	0.262	0.479	0.339	0.227	0.752	0.023	0.152	0.048	1.958	4.802
N Bone formation rate BFR/BS (mcm3/mcm2/yr)	10	17.279	14.015	20.542	16.816	10.752	24.655	20.814	4.562	1.443	0.045	-1.045
O Rel mineral Vol	10	99.443	99.312	99.574	99.351	99.286	99.760	0.033	0.183	0.058	0.991	-0.764
P Surface Density	10	6.085	5.173	6.996	5.853	3.926	8.065	1.624	1.274	0.403	0.290	-0.018
Q Resting Surface	10	87.955	86.591	89.319	88.498	84.674	89.945	3.634	1.906	0.603	-0.526	-1.260
R Surf dens ost seams	10	0.279	0.222	0.337	0.280	0.156	0.400	0.007	0.081	0.026	-0.016	-0.690
S Surf dens ostoid osteoblast interface	10	0.050	0.029	0.071	0.048	0.013	0.104	0.001	0.029	0.009	0.723	-0.148
T Ostoid thickness index	10	11.920	9.817	14.023	11.560	7.768	17.477	8.642	2.940	0.930	0.491	-0.236
U Surface density of Howship's lacunae	10	0.453	0.358	0.547	0.451	0.255	0.756	0.017	0.132	0.042	1.135	2.963
V Surface density of bone osteoclast interface	10	0.048	0.038	0.057	0.046	0.030	0.066	0.000	0.013	0.004	0.014	-1.516
W Total osteoclasts (v)	10	0.395	0.301	0.488	0.388	0.240	0.592	0.017	0.131	0.041	0.142	-1.777
X Bone osteoclasts (TRS)	10	0.921	0.665	1.177	0.856	0.544	1.426	0.128	0.358	0.113	0.284	-1.839
Y Fractional labeled surfaces	10	11.961	10.188	13.734	11.949	7.878	15.010	6.142	2.478	0.784	-0.402	-1.108
Z Fractional double labeled surfaces	10	2.144	1.545	2.744	2.314	0.965	3.524	0.702	0.838	0.265	-0.196	-0.637

Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

### Study 3.2: Groups S20, C: Bone mineral density: Statistical analysis - Mann Whitney U-test

**Mann-Whitney U Test (bmd 1 and 2 femur.sta)**

By variable GR\_ST2

Group 1: 100-Control Group 2: 20mg

	Rank Sum Control	Rank Sum Group 2	U	Z	p-level	adjusted Z	p-level	Valid N Control	Valid N Group 2	2*1sided exact p
<b>BMD_ST_2</b>	117	93	38	0.9071	0.3644	0.9071	0.3644	10	10	0.3930



Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

## Study 3.2: Groups S20, C: QBH: Statistical analysis- Mann Whitney U-test

Mann-Whitney U Test (data histo for graphs.sta)

Group 1: 100-Control Group 2: 101-Simva20

	Rank Sum	Rank Sum	U	Z	p-level	Z		Valid N	Valid N	2*1sided exact p
	Control	Simva20				adjusted	p-level	Control	Simva20	
A Bone Volume (BV/TV)(%)	116	94	39	0.8315	0.4057	0.8315	0.4057	10	10	0.436
B Osteoid volume OV/BV (%)	89	121	34	-1.2095	0.2265	-1.2095	0.2265	10	10	0.247
C Osteoid volume OV/TV (%)	92	118	37	-0.9827	0.3258	-0.9831	0.3256	10	10	0.353
D Osteoid Surface OS/BS (%)	87	123	32	-1.3607	0.1736	-1.3607	0.1736	10	10	0.190
E Osteoblast surface Ob.S/BS (%)	81	129	26	-1.8142	0.0697	-1.8142	0.0697	10	10	0.075
F Osteoid thickness O.Th (mcm)	107.5	102.5	47.5	0.1890	0.8501	0.1891	0.8500	10	10	0.853
G Eroded surface ES/BS (%)	124	86	31	1.4363	0.1509	1.4363	0.1509	10	10	0.165
H Osteoclast surface Oc.S/BS (%)	129	81	26	1.8142	0.0697	1.8142	0.0697	10	10	0.075
J Osteoclast number N.Oc/T.A. (/mm <sup>2</sup> )	115	95	40	0.7559	0.4497	0.7559	0.4497	10	10	0.481
K Mineralising surface MS/BS (%)	136	74	19	2.3434	0.0191	2.3434	0.0191	10	10	0.019
L Osteoid apposition rate OAR xi	115	95	40	0.7559	0.4497	0.7562	0.4495	10	10	0.481
M Mineralisation lag time Mlt (days)	92	118	37	-0.9827	0.3258	-0.9827	0.3258	10	10	0.353
N Bone formation rate BFR/BS (mcm <sup>3</sup> /mcm <sup>2</sup> /yr)	138	72	17	2.4946	0.0126	2.4946	0.0126	10	10	0.011
O Rel mineral Vol	84	126	29	-1.5875	0.1124	-1.5875	0.1124	10	10	0.123

Mann-Whitney U Test (data histo desc stats.sta)

Group 1: 100-Sham Sta Group 2: 101-Simva20

	Rank Sum	Rank Sum	U	Z	p-level	Z		Valid N	Valid N	2*1sided exact p
	Sham Sta	Simva20				adjusted	p-level	Sham Sta	Simva20	
B Osteoid volume OV/BV (%)	131	79	24	1.965	0.049	1.965	0.049	10	10	0.052
C Osteoid volume OV/TV (%)	131.5	78.5	23.5	2.003	0.045	2.005	0.045	10	10	0.043
D Osteoid Surface OS/BS (%)	138	72	17	2.495	0.013	2.495	0.013	10	10	0.011
E Osteoblast surface Ob.S/BS (%)	109	101	46	0.302	0.762	0.302	0.762	10	10	0.796
G Eroded surface ES/BS (%)	117	93	38	0.907	0.364	0.907	0.364	10	10	0.393
H Osteoclast surface Oc.S/BS (%)	93	117	38	-0.907	0.364	-0.907	0.364	10	10	0.393
J Osteoclast number N.Oc/T.A. (/mm <sup>2</sup> )	110	100	45	0.378	0.705	0.378	0.705	10	10	0.739
V Surface density of bone osteoclast interface	101	109	46	-0.302	0.762	-0.302	0.762	10	10	0.796
W Total osteoclasts (v)	109	101	46	0.302	0.762	0.302	0.762	10	10	0.796

Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

### Study 3.2: Groups S20, C: Rat Weights: Descriptive statistics

Descriptive Statistics (statbone 2 weights1.sta)

by GROUP: Control Control

	Valid N	Mean	Confid. -95.000%	Confid. 95.000	Minimum	Maximum	Range	Variance	Std.Dev.	Standard Error	Skewness	Kurtosis
W1	10	247.1	240.17	254.03	230.00	260.00	30.00	93.88	9.69	3.06	-0.70	-0.31

Descriptive Statistics (statbone 2 weights1.sta)

by GROUP: S20 S20

	Valid N	Mean	Confid. -95.000%	Confid. 95.000	Minimum	Maximum	Range	Variance	Std.Dev.	Standard Error	Skewness	Kurtosis
W1	10	241.7	232.22	251.18	220.00	255.00	35.00	175.79	13.26	4.19	-0.63	-0.96



Study 3.2. The effect of simvastatin 20mg/Kg/day administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry, in intact female Sprague-Dawley rats.

### Study 3.2: Groups S20, C: Rat Weights: Statistical analysis - Mann Whitney U-test.

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUP

Group 1: 100-S20 Group 2: 101-Control

	Rank Sum		U	Z	p-level	adjusted	p-level	Valid N		2*1sided exact p
	S20	Control						S20	Control	
<b>W1</b>	94	116	39.00	-0.83	0.41	-0.84	0.40	10.00	10.00	0.44

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUP

Group 1: 100-S20 Group 2: 101-Control

	Rank Sum		U	Z	p-level	adjusted	p-level	Valid N		2*1sided exact p
	S20	Control						S20	Control	
<b>WT_GAIN</b>	91.5	118.5	36.50	-1.02	0.31	-1.03	0.31	10.00	10.00	0.31

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

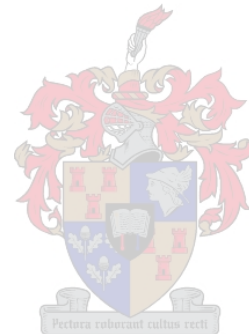
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### Study 3.3: Hard data, Descriptive statistics, Statistical analyses.

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Study 3.3: Groups S20, S10, S5, S1, C: Rat Weights. *Statistical analyses - Mann Whitney U-test*.....249

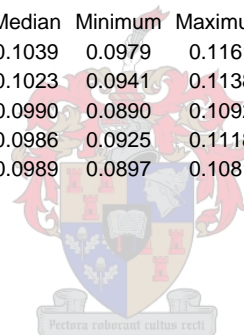


Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: Bone Mineral Density: Descriptive statistics

Descriptive Statistics (bmd 1 and 2 femur.sta)

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Std.Dev.	Standard Error	Skewness	Kurtosis
			-95.000%	+95.000%								
<b>CONTROL</b>	10	0.1053	0.1012	0.1093	0.1039	0.0979	0.1161	0.0000	0.0057	0.0018	0.7839	-0.0845
<b>S20</b>	10	0.1031	0.0991	0.1071	0.1023	0.0941	0.1138	0.0000	0.0056	0.0018	0.5187	0.4303
<b>S10</b>	10	0.1001	0.0952	0.1050	0.0990	0.0890	0.1092	0.0000	0.0069	0.0022	-0.0638	-1.1106
<b>S5</b>	10	0.1024	0.0971	0.1076	0.0986	0.0925	0.1118	0.0001	0.0074	0.0023	0.2722	-1.8984
<b>S1</b>	9	0.0991	0.0943	0.1039	0.0989	0.0897	0.1081	0.0000	0.0063	0.0021	-0.4247	-0.6052



Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: Bone Mineral Density: Statistical analyses - Mann Whitney U-test

**Mann-Whitney U Test (bmd 1 and 2 femur.sta)**

By variable GR\_ST2

**Group 1: 100-Control Group 2: 1mg**

	Rank Sum Control	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Control	Valid N Group 2	2*1sided exact p
<b>BMD_ST_2</b>	121.0000	69.0000	24.0000	1.7146	0.0864	1.7146	0.0864	10	9	0.0947

**Mann-Whitney U Test (bmd 1 and 2 femur.sta)**

By variable GR\_ST2

**Group 1: 100-Control Group 2: 5mg**

	Rank Sum Control	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Control	Valid N Group 2	2*1sided exact p
<b>BMD_ST_2</b>	119.5000	90.5000	35.5000	1.0961	0.2730	1.0965	0.2729	10	10	0.2799

**Mann-Whitney U Test (bmd 1 and 2 femur.sta)**

By variable GR\_ST2

**Group 1: 100-Control Group 2: 10mg**

	Rank Sum Control	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Control	Valid N Group 2	2*1sided exact p
<b>BMD_ST_2</b>	125.5000	84.5000	29.5000	1.5497	0.1212	1.5502	0.1211	10	10	0.1230

**Mann-Whitney U Test (bmd 1 and 2 femur.sta)**

By variable GR\_ST2

**Group 1: 100-Control Group 2: 20mg**

	Rank Sum Control	Rank Sum Group 2	U	Z	p-level	Z adjusted	p-level	Valid N Control	Valid N Group 2	2*1sided exact p
<b>BMD_ST_2</b>	117.0000	93.0000	38.0000	0.9071	0.3644	0.9071	0.3644	10	10	0.3930

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Descriptive statistics

#### Descriptive Statistics (data histo for graphs.sta)

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Standard			
			-95.000%	+95.000%					Std.Dev.	Error	Skewness	Kurtosis
<b>A Bone Volume (BV/TV)(%)</b>	10	22.66	20.36	24.96	22.03	18.89	28.78	10.33	3.21	1.02	0.65	-0.48
<b>B Osteoid volume OV/BV (%)</b>	10	0.92	0.37	1.47	0.82	0.27	2.46	0.59	0.77	0.24	1.32	0.74
<b>C Osteoid volume OV/TV (%)</b>	10	0.20	0.09	0.31	0.19	0.05	0.48	0.02	0.15	0.05	1.01	-0.03
<b>D Osteoid Surface OS/BS (%)</b>	10	6.91	3.53	10.28	5.39	1.96	18.43	22.25	4.72	1.49	1.84	3.88
<b>E Osteoblast surface Ob.S/BS (%)</b>	10	1.75	0.80	2.71	1.49	0.31	4.07	1.79	1.34	0.42	1.08	0.07
<b>F Osteoid thickness O.Th (mcm)</b>	10	6.57	4.77	8.38	6.78	3.09	10.99	6.37	2.52	0.80	0.18	-0.53
<b>G Eroded surface ES/BS (%)</b>	10	9.35	7.85	10.85	9.36	5.74	12.26	4.39	2.10	0.66	-0.30	-0.85
<b>H Osteoclast surface Oc.S/BS (%)</b>	10	1.36	1.01	1.71	1.49	0.72	1.99	0.24	0.49	0.15	-0.06	-1.68
<b>J Osteoclast number N.Oc/T.A. (/mm2)</b>	10	0.11	0.08	0.14	0.12	0.05	0.17	0.00	0.04	0.01	-0.13	-1.43
<b>K Mineralising surface MS/BS (%)</b>	10	7.92	6.84	9.01	7.46	5.88	10.55	2.30	1.52	0.48	0.48	-0.96
<b>L Osteoid apposition rate OAR xi</b>	10	0.71	0.66	0.76	0.70	0.61	0.84	0.01	0.07	0.02	0.40	-0.35
<b>M Mineralisation lag time Mlt (days)</b>	10	0.33	0.23	0.44	0.33	0.14	0.53	0.02	0.15	0.05	-0.01	-1.89
<b>N Bone formation rate BFR/BS (mcm3/mcm2/yr)</b>	10	20.56	17.37	23.75	18.75	15.48	29.41	19.93	4.46	1.41	1.03	0.20
<b>O Rel mineral Vol</b>	10	99.08	98.53	99.63	99.18	97.54	99.73	0.59	0.77	0.24	-1.32	0.74
<b>P Surface Density</b>	10	5.61	5.03	6.19	5.46	4.59	7.24	0.66	0.81	0.26	0.79	0.31
<b>Q Resting Surface</b>	10	83.74	79.63	87.86	85.12	71.00	89.69	33.09	5.75	1.82	-1.34	1.65
<b>R Surf dens ost seams</b>	10	0.38	0.22	0.54	0.33	0.09	0.92	0.05	0.23	0.07	1.60	3.83
<b>S Surf dens ostoid osteoblast interface</b>	10	0.10	0.05	0.15	0.09	0.01	0.22	0.00	0.07	0.02	0.83	-0.35
<b>T Ostoid thickness index</b>	10	12.68	9.46	15.89	13.32	5.93	19.58	20.18	4.49	1.42	-0.12	-0.69
<b>U Surface density of Howship's lacunae</b>	10	0.53	0.41	0.64	0.53	0.33	0.89	0.02	0.15	0.05	1.39	3.09
<b>V Surface density of bone osteoclast interface</b>	10	0.08	0.05	0.10	0.08	0.04	0.14	0.00	0.03	0.01	0.71	0.18
<b>W Total osteoclasts (v)</b>	10	0.65	0.44	0.86	0.64	0.30	1.25	0.08	0.29	0.09	0.82	0.74
<b>X Bone osteoclasts (TRS)</b>	10	1.19	1.00	1.39	1.22	0.86	1.60	0.07	0.27	0.09	0.17	-1.50
<b>Y Fractional labeled surfaces</b>	10	13.19	11.44	14.95	12.62	9.63	17.65	6.03	2.46	0.78	0.39	-0.57
<b>Z Fractional double labeled surfaces</b>	10	2.65	2.06	3.24	2.30	1.76	4.04	0.68	0.83	0.26	0.87	-0.91

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

## Study 3.3: Groups S20, S10,S5, S1, C: QBH: Descriptive statistics

### Descriptive Statistics (data histo for graphs.sta)

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Standard			
			-95.000%	+95.000%					Std.Dev.	Error	Skewness	Kurtosis
<b>A Bone Volume (BV/TV)(%)</b>	10	20.41	17.53	23.30	20.85	12.32	27.00	16.27	4.03	1.28	-0.50	1.02
<b>B Osteoid volume OV/BV (%)</b>	10	0.48	0.13	0.84	0.28	0.11	1.72	0.25	0.50	0.16	2.02	4.04
<b>C Osteoid volume OV/TV (%)</b>	10	0.09	0.03	0.15	0.06	0.02	0.26	0.01	0.08	0.03	1.30	0.62
<b>D Osteoid Surface OS/BS (%)</b>	10	3.96	1.29	6.63	2.77	0.85	13.79	13.98	3.74	1.18	2.39	6.28
<b>E Osteoblast surface Ob.S/BS (%)</b>	10	0.44	0.04	0.84	0.27	0.00	1.95	0.32	0.56	0.18	2.53	7.08
<b>F Osteoid thickness O.Th (mcm)</b>	10	6.55	3.51	9.59	5.49	1.48	15.11	18.07	4.25	1.34	0.79	0.25
<b>G Eroded surface ES/BS (%)</b>	10	6.95	4.18	9.73	5.13	3.85	15.32	15.06	3.88	1.23	1.44	1.15
<b>H Osteoclast surface Oc.S/BS (%)</b>	10	0.74	0.25	1.22	0.46	0.20	2.37	0.46	0.68	0.21	1.95	3.43
<b>J Osteoclast number N.Oc/T.A. (/mm2)</b>	10	0.07	0.03	0.12	0.05	0.02	0.23	0.00	0.06	0.02	1.90	3.46
<b>K Mineralising surface MS/BS (%)</b>	10	5.42	2.65	8.20	4.52	2.46	15.88	15.09	3.88	1.23	2.57	7.26
<b>L Osteoid apposition rate OAR xi</b>	10	0.63	0.53	0.73	0.63	0.39	0.80	0.02	0.14	0.04	-0.44	-0.93
<b>M Mineralisation lag time Mlt (days)</b>	10	0.73	0.31	1.15	0.53	0.12	1.76	0.35	0.59	0.19	0.64	-1.08
<b>N Bone formation rate BFR/BS (mcm3/mcm2/yr)</b>	10	12.14	6.15	18.14	10.34	3.51	34.71	70.25	8.38	2.65	2.52	7.41
<b>O Rel mineral Vol</b>	10	99.52	99.16	99.87	99.72	98.28	99.89	0.25	0.50	0.16	-2.02	4.04
<b>P Surface Density</b>	10	5.50	4.96	6.03	5.61	4.06	6.46	0.57	0.75	0.24	-0.62	-0.26
<b>Q Resting Surface</b>	10	89.09	84.15	94.02	92.20	70.89	93.77	47.60	6.90	2.18	-2.42	6.36
<b>R Surf dens ost seams</b>	10	0.20	0.09	0.31	0.16	0.05	0.56	0.02	0.16	0.05	1.65	2.43
<b>S Surf dens ostoid osteoblast interface</b>	10	0.02	0.01	0.04	0.01	0.00	0.08	0.00	0.02	0.01	1.84	3.93
<b>T Ostoid thickness index</b>	10	13.81	7.35	20.27	13.17	3.10	34.26	81.53	9.03	2.86	1.22	2.19
<b>U Surface density of Howship's lacunae</b>	10	0.37	0.25	0.49	0.29	0.18	0.68	0.03	0.17	0.05	0.91	-0.50
<b>V Surface density of bone osteoclast interface</b>	10	0.04	0.02	0.06	0.02	0.01	0.10	0.00	0.03	0.01	1.46	0.98
<b>W Total osteoclasts (v)</b>	10	0.38	0.19	0.57	0.29	0.14	0.91	0.07	0.26	0.08	1.31	0.82
<b>X Bone osteoclasts (TRS)</b>	10	1.01	0.71	1.32	1.07	0.50	1.58	0.19	0.43	0.14	0.05	-1.79
<b>Y Fractional labeled surfaces</b>	10	9.15	4.44	13.86	7.41	4.43	27.02	43.39	6.59	2.08	2.64	7.61
<b>Z Fractional double labeled surfaces</b>	10	1.70	0.82	2.58	1.33	0.49	4.74	1.52	1.23	0.39	1.93	4.12

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Descriptive statistics

#### Descriptive Statistics (data histo for graphs.sta)

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Standard			
			-95.000%	+95.000%					Std.Dev.	Error	Skewness	Kurtosis
<b>A Bone Volume (BV/TV)(%)</b>	10	22.97	20.99	24.95	22.75	19.58	26.57	7.65	2.77	0.87	0.03	-1.76
<b>B Osteoid volume OV/BV (%)</b>	10	0.42	0.29	0.56	0.40	0.18	0.72	0.04	0.19	0.06	0.33	-1.03
<b>C Osteoid volume OV/TV (%)</b>	10	0.10	0.07	0.13	0.10	0.04	0.18	0.00	0.04	0.01	0.32	0.02
<b>D Osteoid Surface OS/BS (%)</b>	10	3.68	2.54	4.81	3.06	1.86	6.74	2.54	1.59	0.50	1.18	0.41
<b>E Osteoblast surface Ob.S/BS (%)</b>	10	0.67	0.42	0.92	0.55	0.23	1.39	0.12	0.35	0.11	0.91	0.72
<b>F Osteoid thickness O.Th (mcm)</b>	10	6.46	4.06	8.86	5.37	2.34	11.33	11.23	3.35	1.06	0.32	-1.75
<b>G Eroded surface ES/BS (%)</b>	10	5.30	4.47	6.12	5.23	3.91	7.26	1.34	1.16	0.37	0.59	-0.52
<b>H Osteoclast surface Oc.S/BS (%)</b>	10	0.56	0.28	0.84	0.53	0.10	1.48	0.15	0.39	0.12	1.40	3.01
<b>J Osteoclast number N.Oc/T.A. (/mm2)</b>	10	0.06	0.03	0.08	0.05	0.01	0.14	0.00	0.04	0.01	1.18	2.10
<b>K Mineralising surface MS/BS (%)</b>	10	4.54	3.96	5.12	4.67	3.10	5.91	0.66	0.81	0.26	-0.13	-0.08
<b>L Osteoid apposition rate OAR xi</b>	10	1.00	0.91	1.09	0.98	0.78	1.17	0.01	0.12	0.04	-0.40	-0.22
<b>M Mineralisation lag time Mlt (days)</b>	10	0.40	0.24	0.56	0.34	0.11	0.82	0.05	0.22	0.07	0.83	-0.11
<b>N Bone formation rate BFR/BS (mcm3/mcm2/yr)</b>	10	16.71	13.51	19.91	15.69	11.03	25.22	20.05	4.48	1.42	0.55	-0.17
<b>O Rel mineral Vol</b>	10	99.58	99.44	99.71	99.60	99.28	99.82	0.04	0.19	0.06	-0.33	-1.03
<b>P Surface Density</b>	10	5.78	5.25	6.31	5.65	4.85	6.96	0.55	0.74	0.23	0.39	-1.22
<b>Q Resting Surface</b>	10	91.37	90.28	92.46	91.50	88.67	93.61	2.30	1.52	0.48	-0.39	-0.36
<b>R Surf dens ost seams</b>	10	0.21	0.14	0.28	0.18	0.13	0.41	0.01	0.10	0.03	1.34	0.83
<b>S Surf dens ostoid osteoblast interface</b>	10	0.04	0.02	0.05	0.04	0.01	0.09	0.00	0.02	0.01	1.18	2.23
<b>T Ostoid thickness index</b>	10	12.87	7.85	17.89	10.11	4.67	25.57	49.28	7.02	2.22	0.58	-0.91
<b>U Surface density of Howship's lacunae</b>	10	0.31	0.25	0.36	0.28	0.20	0.40	0.01	0.07	0.02	0.24	-1.64
<b>V Surface density of bone osteoclast interface</b>	10	0.03	0.02	0.05	0.03	0.00	0.08	0.00	0.02	0.01	1.01	1.02
<b>W Total osteoclasts (v)</b>	10	0.32	0.18	0.47	0.27	0.06	0.73	0.04	0.20	0.06	0.87	0.53
<b>X Bone osteoclasts (TRS)</b>	10	1.01	0.65	1.37	0.96	0.28	1.89	0.25	0.50	0.16	0.30	-0.53
<b>Y Fractional labeled surfaces</b>	10	7.88	6.88	8.87	7.72	5.86	10.24	1.93	1.39	0.44	0.30	-0.79
<b>Z Fractional double labeled surfaces</b>	10	1.20	0.88	1.53	1.20	0.34	2.02	0.20	0.45	0.14	-0.16	1.23



Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

## Study 3.3: Groups S20, S10,S5, S1, C: QBH: Descriptive statistics

### Descriptive Statistics (data histo for graphs.sta)

S1	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Standard			
			-95.000%	+95.000%					Std.Dev.	Error	Skewness	Kurtosis
<b>A Bone Volume (BV/TV)(%)</b>	9	21.04	17.29	24.79	20.56	14.20	28.32	23.78	4.88	1.63	0.25	-0.99
<b>B Osteoid volume OV/BV (%)</b>	9	0.43	0.10	0.76	0.24	0.09	1.47	0.19	0.43	0.14	2.11	4.81
<b>C Osteoid volume OV/TV (%)</b>	9	0.08	0.03	0.12	0.07	0.02	0.21	0.00	0.06	0.02	1.45	2.41
<b>D Osteoid Surface OS/BS (%)</b>	9	2.86	0.23	5.48	1.71	0.76	11.61	11.66	3.41	1.14	2.59	7.07
<b>E Osteoblast surface Ob.S/BS (%)</b>	9	0.53	0.10	0.96	0.46	0.00	1.75	0.32	0.56	0.19	1.41	2.02
<b>F Osteoid thickness O.Th (mcm)</b>	9	8.51	5.60	11.41	8.24	1.74	13.60	14.33	3.79	1.26	-0.23	0.05
<b>G Eroded surface ES/BS (%)</b>	9	6.78	5.09	8.47	6.05	3.98	10.02	4.84	2.20	0.73	0.31	-1.31
<b>H Osteoclast surface Oc.S/BS (%)</b>	9	0.97	0.64	1.31	0.90	0.42	1.75	0.19	0.44	0.15	0.60	-0.16
<b>J Osteoclast number N.Oc/T.A. (/mm2)</b>	9	0.10	0.06	0.13	0.09	0.04	0.18	0.00	0.05	0.02	0.52	-0.40
<b>K Mineralising surface MS/BS (%)</b>	9	5.04	2.70	7.38	4.33	2.38	12.42	9.30	3.05	1.02	2.05	4.95
<b>L Osteoid apposition rate OAR xi</b>	9	0.51	0.36	0.66	0.55	0.22	0.80	0.04	0.19	0.06	-0.05	-1.00
<b>M Mineralisation lag time Mlt (days)</b>	9	1.22	0.58	1.87	0.97	0.23	2.51	0.70	0.84	0.28	0.67	-1.18
<b>N Bone formation rate BFR/BS (mcm3/mcm2/yr)</b>	9	9.69	4.16	15.23	7.07	2.88	24.80	51.87	7.20	2.40	1.37	1.35
<b>O Rel mineral Vol</b>	9	99.57	99.24	99.90	99.76	98.53	99.91	0.19	0.43	0.14	-2.11	4.81
<b>P Surface Density</b>	9	5.52	4.84	6.19	5.56	4.09	7.22	0.77	0.88	0.29	0.44	1.35
<b>Q Resting Surface</b>	9	90.36	86.43	94.29	92.15	78.38	95.25	26.16	5.11	1.70	-1.86	3.88
<b>R Surf dens ost seams</b>	9	0.14	0.04	0.24	0.10	0.04	0.47	0.02	0.13	0.04	2.31	5.91
<b>S Surf dens ostoid osteoblast interface</b>	9	0.03	0.01	0.04	0.02	0.00	0.07	0.00	0.02	0.01	0.78	0.01
<b>T Ostoid thickness index</b>	9	17.81	11.68	23.93	19.03	3.62	28.22	63.48	7.97	2.66	-0.37	-0.48
<b>U Surface density of Howship's lacunae</b>	9	0.36	0.29	0.43	0.40	0.20	0.46	0.01	0.09	0.03	-0.74	-0.49
<b>V Surface density of bone osteoclast interface</b>	9	0.05	0.04	0.07	0.05	0.02	0.08	0.00	0.02	0.01	-0.28	-1.04
<b>W Total osteoclasts (v)</b>	9	0.52	0.34	0.70	0.54	0.20	0.88	0.05	0.23	0.08	0.06	-1.10
<b>X Bone osteoclasts (TRS)</b>	9	1.38	1.08	1.69	1.20	0.93	2.07	0.15	0.39	0.13	0.65	-0.80
<b>Y Fractional labeled surfaces</b>	9	8.48	4.98	11.98	7.51	4.09	18.79	20.73	4.55	1.52	1.57	2.98
<b>Z Fractional double labeled surfaces</b>	9	1.60	0.28	2.92	1.00	0.40	6.05	2.96	1.72	0.57	2.67	7.54

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Descriptive statistics

#### Descriptive Statistics (data histo for graphs.sta)

C	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Standard			
			-95.000%	+95.000%					Std.Dev.	Error	Skewness	Kurtosis
<b>A Bone Volume (BV/TV)(%)</b>	10	23.97	20.62	27.32	24.34	16.00	29.85	21.98	4.69	1.48	-0.45	-0.79
<b>B Osteoid volume OV/BV (%)</b>	10	0.56	0.43	0.69	0.65	0.24	0.71	0.03	0.18	0.06	-0.99	-0.76
<b>C Osteoid volume OV/TV (%)</b>	10	0.13	0.10	0.17	0.13	0.06	0.20	0.00	0.05	0.02	0.01	-0.68
<b>D Osteoid Surface OS/BS (%)</b>	10	4.66	3.73	5.58	4.68	2.58	7.28	1.68	1.30	0.41	0.61	1.11
<b>E Osteoblast surface Ob.S/BS (%)</b>	10	0.84	0.48	1.19	0.75	0.22	1.72	0.24	0.49	0.16	0.88	-0.08
<b>F Osteoid thickness O.Th (mcm)</b>	10	6.06	4.75	7.37	5.74	3.64	9.07	3.36	1.83	0.58	0.75	-0.33
<b>G Eroded surface ES/BS (%)</b>	10	7.39	6.62	8.16	7.33	5.98	9.38	1.16	1.08	0.34	0.47	-0.30
<b>H Osteoclast surface Oc.S/BS (%)</b>	10	0.80	0.65	0.95	0.82	0.52	1.19	0.05	0.21	0.07	0.41	-0.38
<b>J Osteoclast number N.Oc/T.A. (/mm2)</b>	10	0.07	0.05	0.08	0.07	0.04	0.10	0.00	0.02	0.01	0.20	-1.08
<b>K Mineralising surface MS/BS (%)</b>	10	7.05	5.92	8.18	7.30	4.42	8.81	2.50	1.58	0.50	-0.58	-1.02
<b>L Osteoid apposition rate OAR xi</b>	10	0.67	0.61	0.73	0.67	0.54	0.82	0.01	0.08	0.02	0.42	1.04
<b>M Mineralisation lag time Mlt (days)</b>	10	0.37	0.26	0.48	0.34	0.23	0.75	0.02	0.15	0.05	1.96	4.80
<b>N Bone formation rate BFR/BS (mcm3/mcm2/yr)</b>	10	17.28	14.01	20.54	16.82	10.75	24.65	20.81	4.56	1.44	0.04	-1.05
<b>O Rel mineral Vol</b>	10	99.44	99.31	99.57	99.35	99.29	99.76	0.03	0.18	0.06	0.99	-0.76
<b>P Surface Density</b>	10	6.08	5.17	7.00	5.85	3.93	8.06	1.62	1.27	0.40	0.29	-0.02
<b>Q Resting Surface</b>	10	87.95	86.59	89.32	88.50	84.67	89.95	3.63	1.91	0.60	-0.53	-1.26
<b>R Surf dens ost seams</b>	10	0.28	0.22	0.34	0.28	0.16	0.40	0.01	0.08	0.03	-0.02	-0.69
<b>S Surf dens ostoid osteoblast interface</b>	10	0.05	0.03	0.07	0.05	0.01	0.10	0.00	0.03	0.01	0.72	-0.15
<b>T Ostoid thickness index</b>	10	11.92	9.82	14.02	11.56	7.77	17.48	8.64	2.94	0.93	0.49	-0.24
<b>U Surface density of Howship's lacunae</b>	10	0.45	0.36	0.55	0.45	0.25	0.76	0.02	0.13	0.04	1.13	2.96
<b>V Surface density of bone osteoclast interface</b>	10	0.05	0.04	0.06	0.05	0.03	0.07	0.00	0.01	0.00	0.01	-1.52
<b>W Total osteoclasts (v)</b>	10	0.39	0.30	0.49	0.39	0.24	0.59	0.02	0.13	0.04	0.14	-1.78
<b>X Bone osteoclasts (TRS)</b>	10	0.92	0.67	1.18	0.86	0.54	1.43	0.13	0.36	0.11	0.28	-1.84
<b>Y Fractional labeled surfaces</b>	10	11.96	10.19	13.73	11.95	7.88	15.01	6.14	2.48	0.78	-0.40	-1.11
<b>Z Fractional double labeled surfaces</b>	10	2.14	1.55	2.74	2.31	0.96	3.52	0.70	0.84	0.26	-0.20	-0.64

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Statistical analyses - Mann Whitney U-test

**Mann-Whitney U Test (st2 histo data corelation)**

By variable GROUPS

Group 1: 100-Control Group 2: 101-Simva20

	Rank Sum		U	Z	p-level	Z		Valid N Control	Valid N Simva20	2*1sided exact p
	Control	Simva20				adjusted	p-level			
<b>A Bone Volume (BV/TV)(%)</b>	116	94	39	0.832	0.406	0.832	0.406	10	10	0.436
<b>B Osteoid volume OV/BV (%)</b>	89	121	34	-1.209	0.226	-1.209	0.226	10	10	0.247
<b>C Osteoid volume OV/TV (%)</b>	92	118	37	-0.983	0.326	-0.983	0.326	10	10	0.353
<b>D Osteoid Surface OS/BS (%)</b>	87	123	32	-1.361	0.174	-1.361	0.174	10	10	0.190
<b>E Osteoblast surface Ob.S/BS (%)</b>	81	129	26	-1.814	0.070	-1.814	0.070	10	10	0.075
<b>F Osteoid thickness O.Th (mcm)</b>	97	113	42	-0.605	0.545	-0.605	0.545	10	10	0.579
<b>G Eroded surface ES/BS (%)</b>	78	132	23	-2.041	0.041	-2.041	0.041	10	10	0.043
<b>H Osteoclast surface Oc.S/BS (%)</b>	70	140	15	-2.646	0.008	-2.646	0.008	10	10	0.007
<b>J Osteoclast number N.Oc/T.A. (/mm<sup>2</sup>)</b>	73	137	18	-2.419	0.016	-2.419	0.016	10	10	0.015
<b>K Mineralising surface MS/BS (%)</b>	91	119	36	-1.058	0.290	-1.058	0.290	10	10	0.315
<b>L Osteoid apposition rate OAR xi</b>	89.5	120.5	34.5	-1.172	0.241	-1.173	0.241	10	10	0.247
<b>M Mineralisation lag time Mlt (days)</b>	110	100	45	0.378	0.705	0.378	0.705	10	10	0.739
<b>N Bone formation rate BFR/BS (mcm<sup>3</sup>/mcm<sup>2</sup>/yr)</b>	86	124	31	-1.436	0.151	-1.436	0.151	10	10	0.165
<b>O Rel mineral Vol</b>	121	89	34	1.209	0.226	1.209	0.226	10	10	0.247
<b>P Surface Density</b>	119	91	36	1.058	0.290	1.058	0.290	10	10	0.315
<b>Q Resting Surface</b>	132	78	23	2.041	0.041	2.041	0.041	10	10	0.043
<b>R Surf dens ost seams</b>	86.5	123.5	31.5	-1.398	0.162	-1.399	0.162	10	10	0.165
<b>S Surf dens ostoid osteoblast interface</b>	85	125	30	-1.512	0.131	-1.512	0.131	10	10	0.143
<b>T Ostoid thickness index</b>	100	110	45	-0.378	0.705	-0.378	0.705	10	10	0.739
<b>U Surface density of Howship's lacunae</b>	88	122	33	-1.285	0.199	-1.285	0.199	10	10	0.218
<b>V Surface density of bone ostoclast interface</b>	77	133	22	-2.117	0.034	-2.117	0.034	10	10	0.035
<b>W Total osteoclasts (v)</b>	75.5	134.5	20.5	-2.230	0.026	-2.231	0.026	10	10	0.023
<b>X Bone osteoclasts (TRS)</b>	84	126	29	-1.587	0.112	-1.587	0.112	10	10	0.123
<b>Y Fractional labeled surfaces</b>	90	120	35	-1.134	0.257	-1.134	0.257	10	10	0.280
<b>Z Fractional double labeled surfaces</b>	97	113	42	-0.605	0.545	-0.605	0.545	10	10	0.579

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Statistical analyses - Mann Whitney U-test

Mann-Whitney U Test (st2 histo data corelation)

By variable GROUPS

Group 1: 100-Control Group 2: 102-simva10

	Rank Sum	Rank Sum	U	Z	p-level	Z		Valid N	Valid N	2*1sided exact p
	Control	simva10				adjusted	p-level	Control	simva10	
A Bone Volume (BV/TV)(%)	129	81	26	1.814	0.070	1.814	0.070	10	10	0.075
B Osteoid volume OV/BV (%)	126	84	29	1.587	0.112	1.587	0.112	10	10	0.123
C Osteoid volume OV/TV (%)	127	83	28	1.663	0.096	1.664	0.096	10	10	0.105
D Osteoid Surface OS/BS (%)	129	81	26	1.814	0.070	1.814	0.070	10	10	0.075
E Osteoblast surface Ob.S/BS (%)	137	73	18	2.419	0.016	2.419	0.016	10	10	0.015
F Osteoid thickness O.Th (mcm)	107.5	102.5	47.5	0.189	0.850	0.189	0.850	10	10	0.853
G Eroded surface ES/BS (%)	124	86	31	1.436	0.151	1.436	0.151	10	10	0.165
H Osteoclast surface Oc.S/BS (%)	129	81	26	1.814	0.070	1.814	0.070	10	10	0.075
J Osteoclast number N.Oc/T.A. (/mm2)	115	95	40	0.756	0.450	0.756	0.450	10	10	0.481
K Mineralising surface MS/BS (%)	136	74	19	2.343	0.019	2.343	0.019	10	10	0.019
L Osteoid apposition rate OAR xi	115	95	40	0.756	0.450	0.756	0.450	10	10	0.481
M Mineralisation lag time Mlt (days)	92	118	37	-0.983	0.326	-0.983	0.326	10	10	0.353
N Bone formation rate BFR/BS (mcm3/mcm2/yr)	138	72	17	2.495	0.013	2.495	0.013	10	10	0.011
O Rel mineral Vol	84	126	29	-1.587	0.112	-1.587	0.112	10	10	0.123
P Surface Density	117	93	38	0.907	0.364	0.907	0.364	10	10	0.393
Q Resting Surface	83	127	28	-1.663	0.096	-1.663	0.096	10	10	0.105
R Surf dens ost seams	130	80	25	1.890	0.059	1.890	0.059	10	10	0.063
S Surf dens ostoid osteoblast interface	136	74	19	2.343	0.019	2.343	0.019	10	10	0.019
T Ostoid thickness index	103	107	48	-0.151	0.880	-0.151	0.880	10	10	0.912
U Surface density of Howship's lacunae	122	88	33	1.285	0.199	1.285	0.199	10	10	0.218
V Surface density of bone ostoclast interface	129	81	26	1.814	0.070	1.814	0.070	10	10	0.075
W Total osteoclasts (v)	118	92	37	0.983	0.326	0.983	0.326	10	10	0.353
X Bone osteoclasts (TRS)	100.5	109.5	45.5	-0.340	0.734	-0.340	0.734	10	10	0.739
Y Fractional labeled surfaces	138	72	17	2.495	0.013	2.495	0.013	10	10	0.011
Z Fractional double labeled surfaces	122	88	33	1.285	0.199	1.285	0.199	10	10	0.218

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Statistical analyses - Mann Whitney U-test

Mann-Whitney U Test (st2 histo data corelation)

By variable GROUPS

Group 1: 100-Control Group 2: 103-simva 5

	Rank Sum	Rank Sum	U	Z	p-level	Z		Valid N	Valid N	2*1sided exact p
	Control	simva 5				adjusted	p-level	Control	simva 5	
A Bone Volume (BV/TV)(%)	115	95	40	0.756	0.450	0.756	0.450	10	10	0.481
B Osteoid volume OV/BV (%)	124	86	31	1.436	0.151	1.436	0.151	10	10	0.165
C Osteoid volume OV/TV (%)	127.5	82.5	27.5	1.701	0.089	1.702	0.089	10	10	0.089
D Osteoid Surface OS/BS (%)	127	83	28	1.663	0.096	1.663	0.096	10	10	0.105
E Osteoblast surface Ob.S/BS (%)	114	96	41	0.680	0.496	0.680	0.496	10	10	0.529
F Osteoid thickness O.Th (mcm)	105	105	50	0.000	1.000	0.000	1.000	10	10	1.029
G Eroded surface ES/BS (%)	147	63	8	3.175	0.002	3.175	0.002	10	10	0.001
H Osteoclast surface Oc.S/BS (%)	133	77	22	2.117	0.034	2.117	0.034	10	10	0.035
J Osteoclast number N.Oc/T.A. (/mm2)	120	90	35	1.134	0.257	1.134	0.257	10	10	0.280
K Mineralising surface MS/BS (%)	145	65	10	3.024	0.002	3.024	0.002	10	10	0.002
L Osteoid apposition rate OAR xi	56	154	1	-3.704	0.000	-3.711	0.000	10	10	0.000
M Mineralisation lag time Mlt (days)	106	104	49	0.076	0.940	0.076	0.940	10	10	0.971
N Bone formation rate BFR/BS (mcm3/mcm2/yr)	110	100	45	0.378	0.705	0.378	0.705	10	10	0.739
O Rel mineral Vol	86	124	31	-1.436	0.151	-1.436	0.151	10	10	0.165
P Surface Density	113	97	42	0.605	0.545	0.605	0.545	10	10	0.579
Q Resting Surface	62	148	7	-3.250	0.001	-3.250	0.001	10	10	0.000
R Surf dens ost seams	128	82	27	1.739	0.082	1.739	0.082	10	10	0.089
S Surf dens ostoid osteoblast interface	116	94	39	0.832	0.406	0.832	0.406	10	10	0.436
T Ostoid thickness index	104	106	49	-0.076	0.940	-0.076	0.940	10	10	0.971
U Surface density of Howship's lacunae	141	69	14	2.721	0.007	2.721	0.007	10	10	0.005
V Surface density of bone ostoclast interface	131	79	24	1.965	0.049	1.965	0.049	10	10	0.052
W Total osteoclasts (v)	122.5	87.5	32.5	1.323	0.186	1.323	0.186	10	10	0.190
X Bone osteoclasts (TRS)	101.5	108.5	46.5	-0.265	0.791	-0.265	0.791	10	10	0.796
Y Fractional labeled surfaces	148	62	7	3.250	0.001	3.250	0.001	10	10	0.000
Z Fractional double labeled surfaces	135	75	20	2.268	0.023	2.268	0.023	10	10	0.023

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Statistical analyses - Mann Whitney U-test

#### Mann-Whitney U Test (st2 histo data corelation)

By variable GROUPS

Group 1: 100-Control Group 2: 104-simva 1

	Rank Sum		U	Z	p-level	Z		Valid N Control	Valid N simva 1	2*1sided exact p
	Control	simva 1				adjusted	p-level			
<b>A Bone Volume (BV/TV)(%)</b>	116	74	29	1.306	0.191	1.306	0.191	10	9	0.211
<b>B Osteoid volume OV/BV (%)</b>	123	67	22	1.878	0.060	1.878	0.060	10	9	0.065
<b>C Osteoid volume OV/TV (%)</b>	126	64	19	2.123	0.034	2.127	0.033	10	9	0.035
<b>D Osteoid Surface OS/BS (%)</b>	133	57	12	2.694	0.007	2.694	0.007	10	9	0.006
<b>E Osteoblast surface Ob.S/BS (%)</b>	118	72	27	1.470	0.142	1.470	0.142	10	9	0.156
<b>F Osteoid thickness O.Th (mcm)</b>	78.5	111.5	23.5	-1.755	0.079	-1.759	0.079	10	9	0.079
<b>G Eroded surface ES/BS (%)</b>	112	78	33	0.980	0.327	0.980	0.327	10	9	0.356
<b>H Osteoclast surface Oc.S/BS (%)</b>	89	101	34	-0.898	0.369	-0.898	0.369	10	9	0.400
<b>J Osteoclast number N.Oc/T.A. (/mm2)</b>	83	107	28	-1.388	0.165	-1.388	0.165	10	9	0.182
<b>K Mineralising surface MS/BS (%)</b>	130	60	15	2.449	0.014	2.449	0.014	10	9	0.013
<b>L Osteoid apposition rate OAR xi</b>	124	66	21	1.960	0.050	1.960	0.050	10	9	0.053
<b>M Mineralisation lag time Mlt (days)</b>	67	123	12	-2.694	0.007	-2.694	0.007	10	9	0.006
<b>N Bone formation rate BFR/BS (mcm3/mcm2/yr)</b>	128	62	17	2.286	0.022	2.286	0.022	10	9	0.022
<b>O Rel mineral Vol</b>	77	113	22	-1.878	0.060	-1.878	0.060	10	9	0.065
<b>P Surface Density</b>	113	77	32	1.061	0.288	1.061	0.288	10	9	0.315
<b>Q Resting Surface</b>	72	118	17	-2.286	0.022	-2.286	0.022	10	9	0.022
<b>R Surf dens ost seams</b>	133	57	12	2.694	0.007	2.696	0.007	10	9	0.006
<b>S Surf dens ostoid osteoblast interface</b>	122.5	67.5	22.5	1.837	0.066	1.838	0.066	10	9	0.065
<b>T Ostoid thickness index</b>	75	115	20	-2.041	0.041	-2.041	0.041	10	9	0.043
<b>U Surface density of Howship's lacunae</b>	120	70	25	1.633	0.102	1.633	0.102	10	9	0.113
<b>V Surface density of bone ostoclast interface</b>	94	96	39	-0.490	0.624	-0.490	0.624	10	9	0.661
<b>W Total osteoclasts (v)</b>	85.5	104.5	30.5	-1.184	0.236	-1.184	0.236	10	9	0.243
<b>X Bone osteoclasts (TRS)</b>	75	115	20	-2.041	0.041	-2.041	0.041	10	9	0.043
<b>Y Fractional labeled surfaces</b>	129	61	16	2.368	0.018	2.368	0.018	10	9	0.017
<b>Z Fractional double labeled surfaces</b>	125	65	20	2.041	0.041	2.041	0.041	10	9	0.043

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Statistical analyses - ANOVA; All effects

GROUPS; LS Means (anova histo.sta)

Wilks lambda=.11347, F(44, 132.03)=2.2992,

p=.00015

Effective hypothesis decomposition

		A_BONE_V		A_BONE_V		A_BONE_V		A_BONE_V		B_OSTEOI		B_OSTEOI		B_OSTEOI		B_OSTEOI	
GROUPS		Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%
1	Control	23.971129	1.25795577	21.43588573	26.50637227	0.5566112	0.1481202	0.2580946	0.8551278								
2	Simva20	22.66002484	1.25795577	20.12478157	25.19526811	0.9229872	0.1481202	0.6244705	1.2215038								
3	simva10	20.41238032	1.25795577	17.87713705	22.94762359	0.4837381	0.1481202	0.1852215	0.7822547								
4	simva 5	22.97275915	1.25795577	20.43751589	25.50800242	0.4204743	0.1481202	0.1219577	0.7189909								
5	simva 1	21.03659905	1.32600181	18.364218	23.7089801	0.4263382	0.1561324	0.1116741	0.7410023								

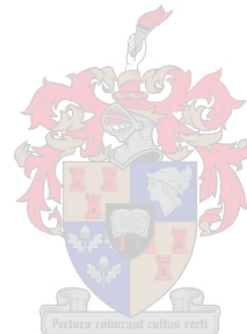
C_OSTEOI	C_OSTEOI	C_OSTEOI	C_OSTEOI	D_OSTEOI	D_OSTEOI	D_OSTEOI	D_OSTEOI	D_OSTEOI	E_OSTEOB	E_OSTEOB	E_OSTEOB	E_OSTEOB	
Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.
0.1314057	0.0277156	0.0755485	0.1872629	4.6567381	1.0194953	2.6020804	6.7113958	0.8361509	0.237416	0.3576704	1.3146315		
0.2024754	0.0277156	0.1466182	0.2583326	6.9052518	1.0194953	4.8505941	8.9599095	1.754879	0.237416	1.2763984	2.2333595		
0.0935893	0.0277156	0.0377322	0.1494465	3.9595466	1.0194953	1.9048889	6.0142043	0.4390165	0.237416	-0.039464	0.9174971		
0.0963887	0.0277156	0.0405315	0.1522459	3.6750944	1.0194953	1.6204367	5.7297521	0.6664853	0.237416	0.1880047	1.1449658		
0.0784112	0.0292148	0.0195325	0.1372898	2.8583533	1.0746424	0.6925539	5.0241527	0.526879	0.2502585	0.0225162	1.0312418		

G_ERODED	G_ERODED	G_ERODED	G_ERODED	H_OSTEOC	H_OSTEOC	H_OSTEOC	H_OSTEOC	H_OSTEOC	J_OSTEOC	J_OSTEOC	J_OSTEOC	J_OSTEOC	
Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.	-95.00%	+95.00%	Mean	Std.Err.
7.388349	0.7329027	5.9112806	8.8654174	0.798863	0.1476738	0.5012459	1.0964801	0.0654654	0.0139785	0.0372937	0.0936371		
9.350524	0.7329027	7.8734556	10.827592	1.3597289	0.1476738	1.0621119	1.657346	0.1143167	0.0139785	0.0861449	0.1424884		
6.9547904	0.7329027	5.477722	8.4318588	0.7390997	0.1476738	0.4414826	1.0367168	0.0738179	0.0139785	0.0456461	0.1019896		
5.2974791	0.7329027	3.8204107	6.7745475	0.5574757	0.1476738	0.2598586	0.8550927	0.0560027	0.0139785	0.0278309	0.0841744		
6.7821358	0.7725473	5.225169	8.3391027	0.9736851	0.1556619	0.6599692	1.2874011	0.0975526	0.0147346	0.067857	0.1272482		

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

<b>N_BONE_F</b>	<b>N_BONE_F</b>	<b>N_BONE_F</b>	<b>N_BONE_F</b>	<b>W_TOTAL</b>	<b>W_TOTAL</b>	<b>W_TOTAL</b>	<b>W_TOTAL</b>	<b>X_BONE_O</b>	<b>X_BONE_O</b>	<b>X_BONE_O</b>	<b>X_BONE_O</b>	
<b>Mean</b>	<b>Std.Err.</b>	<b>-95.00%</b>	<b>+95.00%</b>	<b>Mean</b>	<b>Std.Err.</b>	<b>-95.00%</b>	<b>+95.00%</b>	<b>Mean</b>	<b>Std.Err.</b>	<b>-95.00%</b>	<b>+95.00%</b>	<b>N</b>
17.278585	1.9035316	13.442269	21.114901	0.3947829	0.0728067	0.2480506	0.5415151	0.9212775	0.1260861	0.6671677	1.1753874	10
20.560469	1.9035316	16.724153	24.396784	0.6475376	0.0728067	0.5008053	0.7942698	1.1927945	0.1260861	0.9386846	1.4469043	10
12.141993	1.9035316	8.3056768	15.978308	0.3785835	0.0728067	0.2318512	0.5253157	1.0129474	0.1260861	0.7588376	1.2670573	10
16.710413	1.9035316	12.874097	20.546728	0.3224792	0.0728067	0.175747	0.4692115	1.0060964	0.1260861	0.7519865	1.2602062	10
9.6947419	2.0064984	5.65091	13.738574	0.5232589	0.076745	0.3685895	0.6779283	1.384589	0.1329064	1.1167337	1.6524442	9





Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Statistical analyses - ANOVA; Differences between groups

LSD test; variable A\_BONE\_V (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = 15.825, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	23.971	22.660	20.412	22.973	21.037
1	<b>Control</b>		0.46504517	0.051651751	0.577514127	0.115532
2	<b>Simva20</b>	0.46504517		0.213092341	0.861265161	0.379262
3	<b>simva10</b>	0.05165175	0.21309234		0.157167888	0.734338
4	<b>simva 5</b>	0.57751413	0.86126516	0.157167888		0.295242
5	<b>simva 1</b>	0.11553215	0.37926225	0.734338006	0.295242367	

LSD test; variable B\_OSTEOI (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .21940, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	.55661	.92299	.48374	.42047	.42634
1	<b>Control</b>		0.08725887	0.729584124	0.519135745	0.548076
2	<b>Simva20</b>	0.08725887		0.041782097	0.020746647	0.025779
3	<b>simva10</b>	0.72958412	0.0417821		0.764064997	0.790938
4	<b>simva 5</b>	0.51913575	0.02074665	0.764064997		0.978386
5	<b>simva 1</b>	0.54807647	0.02577865	0.790937524	0.978386106	

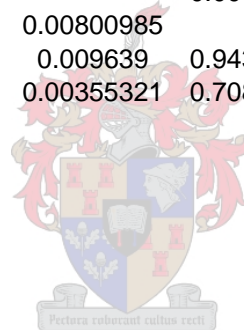
Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

LSD test; variable C\_OSTEOI (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .00768, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	.13141	.20248	.09359	.09639	.07841
<b>1</b>	<b>Control</b>		0.07662632	0.339915057	0.376511019	0.194995
<b>2</b>	<b>Simva20</b>	0.07662632		0.008009853	0.009638998	0.003553
<b>3</b>	<b>simva10</b>	0.33991506	0.00800985		0.943387262	0.708051
<b>4</b>	<b>simva 5</b>	0.37651102	0.009639	0.943387262		0.657479
<b>5</b>	<b>simva 1</b>	0.19499453	0.00355321	0.708051261	0.657478835	



Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Statistical analyses - ANOVA; Differences between groups

LSD test; variable D\_OSTEOI (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = 10.394, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	4.6567	6.9053	3.9595	3.6751	2.8584
1	<b>Control</b>		0.12603417	0.631094842	0.499531762	0.231199
2	<b>Simva20</b>	0.12603417		0.047060528	0.030164381	0.009027
3	<b>simva10</b>	0.63109484	0.04706053		0.844507761	0.461191
4	<b>simva 5</b>	0.49953176	0.03016438	0.844507761		0.584169
5	<b>simva 1</b>	0.23119928	0.00902693	0.461190527	0.584168958	

LSD test; variable E\_OSTEOB (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .56366, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	.83615	1.7549	.43902	.66649	.52688
1	<b>Control</b>		0.00892741	0.24323918	0.615854114	0.374838
2	<b>Simva20</b>	0.00892741		0.000306653	0.002268606	0.000905
3	<b>simva10</b>	0.24323918	0.00030665		0.501648172	0.800137
4	<b>simva 5</b>	0.61585411	0.00226861	0.501648172		0.687655
5	<b>simva 1</b>	0.37483787	0.00090461	0.800137477	0.687654631	

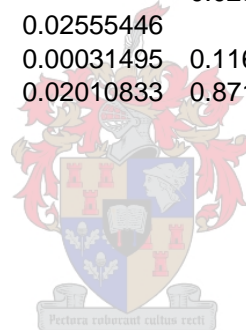
Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

LSD test; variable G\_ERODED (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = 5.3715, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	7.3883	9.3505	6.9548	5.2975	6.7821
<b>1</b>	<b>Control</b>		0.06493148	0.67776341	0.04979253	0.572063
<b>2</b>	<b>Simva20</b>	0.06493148		0.025554459	0.00031495	0.020108
<b>3</b>	<b>simva10</b>	0.67776341	0.02555446		0.116982489	0.871942
<b>4</b>	<b>simva 5</b>	0.04979253	0.00031495	0.116982489		0.170259
<b>5</b>	<b>simva 1</b>	0.5720632	0.02010833	0.871942022	0.170258959	



Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Statistical analyses - ANOVA; Differences between groups

LSD test; variable H\_OSTEOC (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .21808, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	.79886	1.3597	.73910	.55748	.97369
1	<b>Control</b>		0.0101729	0.776095089	0.253986418	0.41959
2	<b>Simva20</b>	0.0101729		0.004785597	0.000388834	0.078849
3	<b>simva10</b>	0.77609509	0.0047856		0.389200205	0.280209
4	<b>simva 5</b>	0.25398642	0.00038883	0.389200205		0.058833
5	<b>simva 1</b>	0.41958981	0.07884893	0.280208972	0.058833285	

LSD test; variable J\_OSTEOC (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .00195, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	.06547	.11432	.07382	.05600	.09755
1	<b>Control</b>		0.0174105	0.674707662	0.634538064	0.121304
2	<b>Simva20</b>	0.0174105		0.046490395	0.00507733	0.413597
3	<b>simva10</b>	0.67470766	0.04649039		0.372392993	0.248854
4	<b>simva 5</b>	0.63453806	0.00507733	0.372392993		0.046786
5	<b>simva 1</b>	0.12130351	0.41359698	0.248854223	0.046785974	

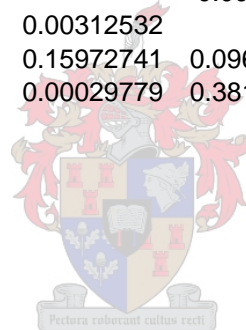
Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

LSD test; variable N\_BONE\_F (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = 36.234, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	17.279	20.560	12.142	16.710	9.6947
<b>1</b>	<b>Control</b>		0.2292925	0.062917376	0.83381519	0.008795
<b>2</b>	<b>Simva20</b>	0.2292925		0.00312532	0.159727408	0.000298
<b>3</b>	<b>simva10</b>	0.06291738	0.00312532		0.096753993	0.381055
<b>4</b>	<b>simva 5</b>	0.83381519	0.15972741	0.096753993		0.014816
<b>5</b>	<b>simva 1</b>	0.00879539	0.00029779	0.381055001	0.014816113	



Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10,S5, S1, C: QBH: Statistical analyses - ANOVA; Differences between groups

LSD test; variable W\_TOTAL (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .05301, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	.39478	.64754	.37858	.32248	.52326
1	<b>Control</b>		0.01812132	0.875704117	0.486239619	0.231037
2	<b>Simva20</b>	0.01812132		0.012263802	0.00287677	0.246387
3	<b>simva10</b>	0.87570412	0.0122638		0.588580082	0.178374
4	<b>simva 5</b>	0.48623962	0.00287677	0.588580082		0.06427
5	<b>simva 1</b>	0.23103714	0.2463873	0.178373887	0.064270408	

LSD test; variable X\_BONE\_O (anova histo.sta)

Probabilities for Post Hoc Tests

Error: Between MS = .15898, df = 44.000

		{1}	{2}	{3}	{4}	{5}
	<b>GROUPS</b>	.92128	1.1928	1.0129	1.0061	1.3846
1	<b>Control</b>		0.13498878	0.609757114	0.636659339	0.015098
2	<b>Simva20</b>	0.13498878		0.318678727	0.300805053	0.300854
3	<b>simva10</b>	0.60975711	0.31867873		0.969525346	0.048575
4	<b>simva 5</b>	0.63665934	0.30080505	0.969525346		0.044745
5	<b>simva 1</b>	0.01509822	0.30085386	0.048575488	0.04474526	

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10, S5, S1, C: Rat Weights. *Descriptive statistics.*

Descriptive Statistics (statbone 2 weights1.sta)

by GROUPS: **Simva 1m Simva 1mg**

			Confid.	Confid.					Standard		
	Valid N	Mean	-95.000%	95.000	Minimum	Maximum	Variance	Std.Dev.	Error	Skewness	Kurtosis
<b>W1</b>	10	239.5	231.74	247.26	222.00	255.00	117.61	10.84	3.43	-0.14	-1

Descriptive Statistics (statbone 2 weights1.sta)

by GROUPS: **Simva 5m Simva 5mg**

			Confid.	Confid.					Standard		
	Valid N	Mean	-95.000%	95.000	Minimum	Maximum	Variance	Std.Dev.	Error	Skewness	Kurtosis
<b>W1</b>	10	240.3	231.51	249.09	224.00	255.00	150.90	12.28	3.88	-0.05	-2

Descriptive Statistics (statbone 2 weights1.sta)

by GROUPS: **Simva 10 Simva 10mg**

			Confid.	Confid.					Standard		
	Valid N	Mean	-95.000%	95.000	Minimum	Maximum	Variance	Std.Dev.	Error	Skewness	Kurtosis
<b>W1</b>	10	239.9	230.54	249.26	219.00	264.00	171.21	13.08	4.14	0.54	0

Descriptive Statistics (statbone 2 weights1.sta)

by GROUPS: **Simva 20 Simva 20mg**

			Confid.	Confid.					Standard		
	Valid N	Mean	-95.000%	95.000	Minimum	Maximum	Variance	Std.Dev.	Error	Skewness	Kurtosis
<b>W1</b>	10	241.7	232.22	251.18	220.00	255.00	175.79	13.26	4.19	-0.63	-1

Descriptive Statistics (statbone 2 weights1.sta)

by GROUPS: **Control Control**

			Confid.	Confid.					Standard		
	Valid N	Mean	-95.000%	95.000	Minimum	Maximum	Variance	Std.Dev.	Error	Skewness	Kurtosis
<b>W1</b>	10	247.1	240.17	254.03	230.00	260.00	93.88	9.69	3.06	-0.70	0



Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10, S5, S1, C: Rat Weights. *Statistical analyses - Mann Whitney U-test*

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 100-Simva 1m Group 2: 101-Simva 5m**

	Rank Sum Simva 1m	Rank Sum Simva 5m	U	Z	p-level	Z	Valid N Simva 1m	Valid N Simva 5m	2*1sided exact p
<b>W1</b>	99.00	111.00	44.00	-0.45	0.65	-0.45	10.00	10.00	0.68

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 100-Simva 1m Group 2: 102-Simva 10**

	Rank Sum Simva 1m	Rank Sum Simva 10	U	Z	p-level	Z	Valid N Simva 1m	Valid N Simva 10	2*1sided exact p
<b>W1</b>	103.00	107.00	48.00	-0.15	0.88	-0.15	10.00	10.00	0.91

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 100-Simva 1m Group 2: 103-Simva 20**

	Rank Sum Simva 1m	Rank Sum Simva 20	U	Z	p-level	Z	Valid N Simva 1m	Valid N Simva 20	2*1sided exact p
<b>W1</b>	98.50	111.50	43.50	-0.49	0.62	-0.49	10.00	10.00	0.63

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 100-Simva 1m Group 2: 104-Control**

	Rank Sum Simva 1m	Rank Sum Control	U	Z	p-level	Z	Valid N Simva 1m	Valid N Control	2*1sided exact p
<b>W1</b>	82.50	127.50	27.50	-1.70	0.09	-1.70	10.00	10.00	0.09

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10, S5, S1, C: Rat Weights. *Statistical analyses - Mann Whitney U-test*

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 101-Simva 5m Group 2: 102-Simva 10**

	Rank Sum Simva 5m	Rank Sum Simva 10	U	Z	p-level	Z	Valid N Simva 5m	Valid N Simva 10	2*1sided exact p
<b>W1</b>	105.00	105.00	50.00	0.00	1.00	0.00	10.00	10.00	1.03

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 101-Simva 5m Group 2: 103-Simva 20**

	Rank Sum Simva 5m	Rank Sum Simva 20	U	Z	p-level	Z	Valid N Simva 5m	Valid N Simva 20	2*1sided exact p
<b>W1</b>	103.50	106.50	48.50	-0.11	0.91	-0.11	10.00	10.00	0.91

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 101-Simva 5m Group 2: 104-Control**

	Rank Sum Simva 5m	Rank Sum Control	U	Z	p-level	Z	Valid N Simva 5m	Valid N Control	2*1sided exact p
<b>W1</b>	89.50	120.50	34.50	-1.17	0.24	-1.17	10.00	10.00	0.25

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 102-Simva 10 Group 2: 103-Simva 20**

	Rank Sum Simva 10	Rank Sum Simva 20	U	Z	p-level	Z	Valid N Simva 10	Valid N Simva 20	2*1sided exact p
<b>W1</b>	98.50	111.50	43.50	-0.49	0.62	-0.49	10.00	10.00	0.63

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10, S5, S1, C: Rat Weights. Statistical analyses - Mann Whitney U-test

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

Group 1: 102-Simva 10 Group 2: 104-Control

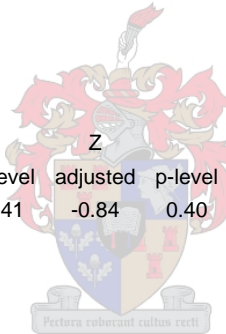
	Rank Sum		U	Z	p-level	Z		Valid N		2*1sided exact p
	Simva 10	Control				adjusted	p-level	Simva 10	Control	
<b>W1</b>	87.50	122.50	32.50	-1.32	0.19	-1.33	0.19	10.00	10.00	0.19

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

Group 1: 103-Simva 20 Group 2: 104-Control

	Rank Sum		U	Z	p-level	Z		Valid N		2*1sided exact p
	Simva 20	Control				adjusted	p-level	Simva 20	Control	
<b>W1</b>	94.00	116.00	39.00	-0.83	0.41	-0.84	0.40	10.00	10.00	0.44



Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

Group 1: 100-Simva 1m Group 2: 101-Simva 5m

	Rank Sum		U	Z	p-level	Z		Valid N		2*1sided exact p
	Simva 1m	Simva 5m				adjusted	p-level	Simva 1m	Simva 5m	
<b>WT_GAIN</b>	98.00	112.00	43.00	-0.53	0.60	-0.53	0.60	10.00	10.00	0.63

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

Group 1: 100-Simva 1m Group 2: 102-Simva 10

	Rank Sum		U	Z	p-level	Z		Valid N		2*1sided exact p
	Simva 1m	Simva 10				adjusted	p-level	Simva 1m	Simva 10	
<b>WT_GAIN</b>	82.00	128.00	27.00	-1.74	0.08	-1.74	0.08	10.00	10.00	0.09

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10, S5, S1, C: Rat Weights. Statistical analyses - Mann Whitney U-test

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 100-Simva 1m Group 2: 103-Simva 20**

	Rank Sum	Rank Sum				Z		Valid N	Valid N	2*1sided
	Simva 1m	Simva 20	U	Z	p-level	adjusted	p-level	Simva 1m	Simva 20	exact p
<b>WT_GAIN</b>	90.00	120.00	35.00	-1.13	0.26	-1.14	0.25	10.00	10.00	0.28

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 100-Simva 1m Group 2: 104-Control**

	Rank Sum	Rank Sum				Z		Valid N	Valid N	2*1sided
	Simva 1m	Control	U	Z	p-level	adjusted	p-level	Simva 1m	Control	exact p
<b>WT_GAIN</b>	77.50	132.50	22.50	-2.08	0.04	-2.08	0.04	10.00	10.00	0.04

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 101-Simva 5m Group 2: 102-Simva 10**

	Rank Sum	Rank Sum				Z		Valid N	Valid N	2*1sided
	Simva 5m	Simva 10	U	Z	p-level	adjusted	p-level	Simva 5m	Simva 10	exact p
<b>WT_GAIN</b>	94.50	115.50	39.50	-0.79	0.43	-0.79	0.43	10.00	10.00	0.44

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 101-Simva 5m Group 2: 103-Simva 20**

	Rank Sum	Rank Sum				Z		Valid N	Valid N	2*1sided
	Simva 5m	Simva 20	U	Z	p-level	adjusted	p-level	Simva 5m	Simva 20	exact p
<b>WT_GAIN</b>	102.00	108.00	47.00	-0.23	0.82	-0.23	0.82	10.00	10.00	0.85

Study 3.3 The effect of different dosages of simvastatin (20mg/Kg/day, 10mg/Kg/day, 5mg/Kg/day and 1mg/Kg/day) administered for 12 weeks, on bone mineral density and quantitative bone histomorphometry in intact female Sprague-Dawley rats.

### Study 3.3: Groups S20, S10, S5, S1, C: Rat Weights. Statistical analyses - Mann Whitney U-test

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 101-Simva 5m Group 2: 104-Control**

	Rank Sum Simva 5m	Rank Sum Control	U	Z	p-level	Z	p-level	Valid N Simva 5m	Valid N Control	2*1sided exact p
<b>WT_GAIN</b>	94.00	116.00	39.00	-0.83	0.41	-0.83	0.41	10.00	10.00	0.44

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 102-Simva 10 Group 2: 103-Simva 20**

	Rank Sum Simva 10	Rank Sum Simva 20	U	Z	p-level	Z	p-level	Valid N Simva 10	Valid N Simva 20	2*1sided exact p
<b>WT_GAIN</b>	114.50	95.50	40.50	0.72	0.47	0.72	0.47	10.00	10.00	0.48

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 102-Simva 10 Group 2: 104-Control**

	Rank Sum Simva 10	Rank Sum Control	U	Z	p-level	Z	p-level	Valid N Simva 10	Valid N Control	2*1sided exact p
<b>WT_GAIN</b>	104.50	105.50	49.50	-0.04	0.97	-0.04	0.97	10.00	10.00	0.97

Mann-Whitney U Test (statbone 2 weights1.sta)

By variable GROUPS

**Group 1: 103-Simva 20 Group 2: 104-Control**

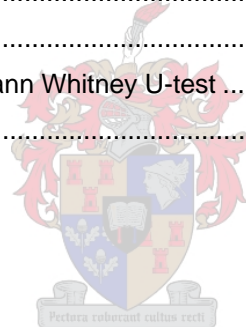
	Rank Sum Simva 20	Rank Sum Control	U	Z	p-level	Z	p-level	Valid N Simva 20	Valid N Control	2*1sided exact p
<b>WT_GAIN</b>	91.50	118.50	36.50	-1.02	0.31	-1.03	0.31	10.00	10.00	0.31

Study 3.4 The effect atorvastatin 2.5mg/Kg/day and pravastatin, administered for 12 weeks, on bone mineral density in intact female Sprague-Dawley rats.

## **Appendix D 3.4**

### **Study 3.4: Hard data, Descriptive statistics, Statistical analyses.**

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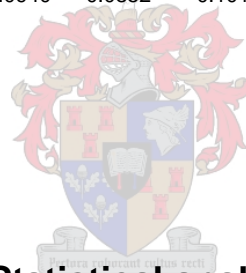


Study 3.4 The effect atorvastatin 2.5mg/Kg/day and pravastatin, administered for 12 weeks, on bone mineral density in intact female Sprague-Dawley rats.

### Study 3.4: Groups A, C: Bone Mineral Density: Descriptive statistics

Descriptive Statistics (bmd 1 and 2 femur.sta)

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Std.Dev.	Standard		
			-95.000%	+95.000%						Error	Skewness	Kurtosis
CONTROL	10	0.1053	0.1012	0.1093	0.1039	0.0979	0.1161	0.0000	0.0057	0.0018	0.7839	-0.0845
A	10	0.0942	0.0909	0.0975	0.0940	0.0852	0.1014	0.0000	0.0046	0.0015	-0.2180	0.7944



### Study 3.4: Groups A, C: Bone Mineral Density: Statistical analyses - Mann Whitney U-test

Mann-Whitney U Test (bmd 1 and 2 femur.sta)

By variable GR\_ST2

Group 1: 100-Control Group 2: 101-Atorva

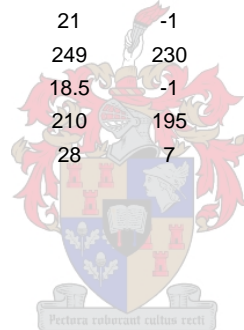
	Rank Sum		U	Z	p-level	adjusted p-level	Valid N Control	Valid N Atorva	2*1sided exact p	
	Control	Atorva								
BMD_ST_2	150.5	59.5	4.5	3.4395	0.0006	3.4408	0.0006	10	10	0.0001

Study 3.4 The effect atorvastatin 2.5mg/Kg/day and pravastatin, administered for 12 weeks, on bone mineral density in intact female Sprague-Dawley rats.

### Study 3.4: Groups A, C: Rat Weights: Descriptive statistics

Descriptive Statistics (statbone 2 weights1.sta)

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Std.Dev.	Standard		
			-95.000%	+95.000%					Error	Skewness	Kurtosis
WT_GAIN	20	25.05	17.59	32.51	21	-1	55	15.95	3.57	0.34	-1.05
W1CONTR	10	247.1	240.17	254.03	249	230	260	9.69	3.06	-0.70	-0.31
WT_GN_C	10	21.5	10.75	32.25	18.5	-1	47	15.03	4.75	0.59	-0.19
W1AT	10	212.4	203.24	221.56	210	195	230	12.80	4.05	0.10	-1.58
WT_GN_A	10	28.6	16.56	40.64	28	7	55	16.83	5.32	0.11	-1.46





Study 3.4 The effect atorvastatin 2.5mg/Kg/day and pravastatin, administered for 12 weeks, on bone mineral density in intact female Sprague-Dawley rats.

### Study 3.4: Groups P, C: Bone Mineral Density: Descriptive statistics

Descriptive Statistics (bmd 1 and 2 femur.sta)

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Std.Dev.	Standard		Skewness	Kurtosis
			-95.000%	+95.000%						Error			
<b>CONTROL</b>	10	0.1053	0.1012	0.1093	0.1039	0.0979	0.1161	0.0000	0.0057	0.0018	0.7839	-0.0845	
<b>P</b>	10	0.0965	0.0918	0.1012	0.0980	0.0802	0.1022	0.0000	0.0065	0.0021	-1.9472	4.4711	



### Study 3.4: Groups P, C: Bone Mineral Density: Statistical analyses - Mann Whitney U-test

**Mann-Whitney U Test (bmd 1 and 2 femur.sta)**

By variable GR\_ST2

Group 1: 100-**Control** Group 2: 102-**Prava**

	Rank Sum		U	Z	p-level	adjusted	p-level	Valid N	Valid N	2*1sided
	Control	Prava								
<b>BMD_ST_2</b>	142.5	67.5	12.5	2.8347	0.0046	2.8358	0.0046	10	10	0.0029

Study 3.4 The effect atorvastatin 2.5mg/Kg/day and pravastatin, administered for 12 weeks, on bone mineral density in intact female Sprague-Dawley rats.

### Study 3.4: Groups P, C: Rat Weights: Descriptive statistics

Descriptive Statistics (statbone 2 weights1.sta)

	Valid N	Mean	Confid.		Median	Minimum	Maximum	Variance	Std.Dev.	Standard		
			-95.000%	+95.000%						Error	Skewness	Kurtosis
WT_GAIN	20	25.95	16.23	35.67	21	-9	70	431.21	20.77	4.64	0.54	0.01
W1CONTR	10	247.1	240.17	254.03	249	230	260	93.88	9.69	3.06	-0.70	-0.31
WT_GN_C	10	21.5	10.75	32.25	18.5	-1	47	225.83	15.03	4.75	0.59	-0.19
W1PR	10	226.4	208.04	244.76	228.5	181	263	658.93	25.67	8.12	-0.44	-0.46
WT_GN_PR	10	30.4	12.30	48.50	27	-9	70	640.49	25.31	8.00	0.16	-0.53

