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**Studies of the Pathogenesis and Treatment  
of Secondary Osteoporosis**

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**A thesis submitted in partial fulfilment of the requirements  
for the degree of Doctor of Philosophy,  
Faculty of Medical and Health Sciences,  
The University of Auckland, 2006.**

## **Abstract:**

### **Background:**

Osteoporosis commonly affects older men and women. Frequently, it is caused or exacerbated by a disease or treatment (termed secondary osteoporosis). The aim of this thesis is to explore aspects of the pathogenesis and treatment of three conditions that may cause or contribute to osteoporosis: human immunodeficiency virus (HIV) infection, primary hyperparathyroidism (PHPT), and vitamin D insufficiency. Each of these conditions is potentially linked to bone metabolism via associations with nutritional status, which in turn is a key regulator of bone mass and fracture risk.

### **Methods:**

Eight studies were performed:

1. Cross-sectional comparison of bone mineral density (BMD) in HIV-infected men and age-matched controls.
2. Two-year randomised controlled trial of the effects of annual zoledronate on BMD in HIV-infected men.
3. Longitudinal comparison of the change in BMD over two years in HIV-infected men not receiving skeletal therapy and controls.
4. Meta-analysis of body weight in patients with PHPT.
5. Cross-sectional analysis of the relationship between parathyroid hormone and body weight in post-menopausal women.
6. Cross-sectional analysis of the determinants of 25-hydroxyvitamin D (25OHD) in post-menopausal women and middle-aged and older men.
7. Cross-sectional analysis of the effects of fat mass and seasonal variation on diagnosis of vitamin D sufficiency.
8. Cross-sectional analysis of the relationship between vitamin D binding protein and body weight in older men and women.

## **Results:**

1. HIV-infected men were 6.3 kg lighter than controls, and after adjustment for this weight difference, did not have lower BMD than controls.
2. Annual intravenous zoledronate caused substantial increases in BMD in HIV-infected men over two years.
3. HIV-infected men did not have accelerated bone loss over time compared to controls.
4. Patients with PHPT are on average 3.1-3.3 kg heavier than age-matched controls.
5. Fat mass is an important determinant of parathyroid hormone levels.
6. The major determinants of 25OHD levels in men and women are surrogate measures of ultraviolet-B exposure, and fat mass, but men have higher 25OHD levels throughout the year than women.
7. Thresholds for diagnosis of vitamin D sufficiency vary by season and amount of fat mass.
8. Vitamin D binding protein does not mediate the relationships between 25OHD and age, gender or weight.

## **Conclusions:**

Uncomplicated HIV infection is not associated with low BMD at baseline or accelerated bone loss over time. There are important relationships between body weight and PHPT, and among fat mass, parathyroid hormone and 25OHD that have significance both for the pathogenesis of PHPT and vitamin D sufficiency, and for the clinical diagnosis and treatment of these conditions.

## Preface:

Osteoporosis affects a significant number of older men and women. Osteoporosis is defined by the WHO as “a systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fractures” [1]. In Caucasian populations, approximately 50% of post-menopausal women and 20% of men older than 50 years will experience at least one fragility fracture in their remaining lifetime [2]. In Caucasian populations, the lifetime risk for fragility fracture of the hip is 14% for women and 3% for men, and for fragility fracture of the spine is 28% for women and 12% for men [3]. In the majority of cases of osteoporosis, the bone loss is due to the normal ageing process, in which case it is termed primary or idiopathic osteoporosis. However, in up to 20-30% of post-menopausal women, 50% of pre-menopausal women, and more than 50% of men, osteoporosis is caused or exacerbated by a disease or treatment, in which case it is termed secondary osteoporosis [4]. Currently, there are more than 50 recognised causes of secondary osteoporosis [4]. Since there are very large numbers of people affected by osteoporosis and many of these cases of osteoporosis will be due to secondary causes, secondary osteoporosis is a significant health issue.

In 2000, Tebas et al. published the first report which suggested HIV or treatment of HIV with antiretroviral agents may cause osteoporosis [5]. In contrast, PHPT has been recognised to cause osteoporosis since the earliest descriptions of patients with osteitis fibrosa cystica [6], and vitamin D deficiency has also been known for many years to cause osteopenia and osteomalacia [7]. PTH and vitamin D are essential hormones in calcium homeostasis, and their roles in calcium metabolism are interlinked [8, 9]. Therefore, diseases with abnormal levels of serum PTH are likely to impact upon vitamin D metabolism, and, conversely, diseases with abnormal levels of serum vitamin D metabolites are likely to impact upon parathyroid gland function. Thus, in any consideration of PHPT or vitamin D insufficiency, it is important to consider the roles of PTH and vitamin D and its metabolites. Recent evidence has suggested that both PHPT and vitamin D insufficiency are associated with increased body weight. Since body weight is a major determinant of BMD and an important risk factor for osteoporotic fractures [10], and both PHPT and vitamin D insufficiency are important causes of secondary osteoporosis, it is important to explore the role of body weight in the pathogenesis of PHPT and vitamin D insufficiency.

The purpose of this thesis is to explore aspects of the pathogenesis and treatment of secondary osteoporosis in association with three conditions: HIV, PHPT and vitamin D insufficiency. In Chapters 1-4, I will review the association between HIV and osteoporosis, and then report and discuss the results of a cross-sectional study and a longitudinal study of BMD in HIV-infected men in comparison to age-matched healthy controls, and an intervention study on the effect of the potent bisphosphonate zoledronate on BMD in HIV-infected men. In Chapters 5-10, I will review the relationship between PTH, vitamin D, body weight and BMD, and then report and discuss the results of five studies that explore aspects of the relationships between PTH and body weight, vitamin D metabolites and body weight, and the determinants of PTH and vitamin D metabolites in a variety of population groups.

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## **List of Publications:**

### **Chapter 2:**

Bolland MJ, Grey AB, Horne AM, Briggs SE, Thomas MG, Ellis-Pegler RB, Woodhouse AF, Gamble GD, Reid IR. Bone mineral density is not reduced in HIV-infected Caucasian men treated with highly active antiretroviral therapy. *Clin Endocrinol (Oxf)*. 2006; 65:191-7

### **Chapter 3:**

Bolland MJ, Grey AB, Horne AM, Briggs SE, Thomas MG, Ellis-Pegler RB, Woodhouse AF, Gamble GD, Reid IR. Annual zoledronate increases bone density in highly active antiretroviral therapy-treated human immunodeficiency virus-infected men: a randomized controlled trial. *J Clin Endocrinol Metab*. 2007; 92:1283-8.

### **Chapter 4:**

Bolland MJ, Grey AB, Horne AM, Briggs SE, Thomas MG, Ellis-Pegler RB, Woodhouse AF, Gamble GD, Reid IR. Bone mineral density remains stable in HAART-treated HIV-infected men over 2 years. *Clin Endocrinol (Oxf)*. 2007; 67:270-5.

### **Chapter 6:**

Bolland MJ, Grey AB, Gamble GD, Reid IR. Association between primary hyperparathyroidism and increased body weight: a meta-analysis. *J Clin Endocrinol Metab*. 2005; 90:1525-30

### **Chapter 7:**

Bolland MJ, Grey AB, Ames RW, Horne AM, Gamble GD, Reid IR. Fat mass is an important predictor of parathyroid hormone levels in postmenopausal women. *Bone*. 2006; 38:317-21

### **Chapter 8:**

Lucas JA, Bolland MJ, Grey AB, Ames RW, Mason BH, Horne AM, Gamble GD, Reid IR. Determinants of vitamin D status in older women living in a subtropical climate. *Osteoporos Int*. 2005; 16:1641-8

Bolland MJ, Grey AB, Ames RW, Mason BH, Horne AM, Gamble GD, Reid IR. Determinants of vitamin D status in older men living in a subtropical climate. *Osteoporos Int.* 2006; 17:1742-8

### **Chapter 9:**

Bolland MJ, Grey AB, Ames RW, Mason BH, Horne AM, Gamble GD, Reid IR. The effects of seasonal variation of 25-hydroxyvitamin D and fat mass on diagnosis of vitamin D sufficiency. *Am J Clin Nutr.* 2007; 86:959-64.

### **Chapter 10:**

Bolland MJ, Grey AB, Ames RW, Horne AM, Mason BH, Wattie DJ, Gamble GD, Bouillon R, Reid IR. Age-, gender-, and weight-related effects on levels of 25-hydroxyvitamin D are not mediated by vitamin D binding protein. *Clin Endocrinol (Oxf).* 2007; 67:259-64.

## Abbreviations:

1,25(OH) <sub>2</sub> D	1,25-dihydroxyvitamin D
25OHD	25-hydroxyvitamin D
AIDS	Acquired immunodeficiency syndrome
ALP	Alkaline phosphatase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
bsALP	Bone specific alkaline phosphatase
CI	Confidence interval
CTx	β-C-terminal telopeptide of type I collagen
CV	Coefficient of variation
DPD	Deoxypyridinoline
DXA	Dual energy x-ray absorptiometer
ELISA	Enzyme linked immunosorbent assay
GGT	γ-Glutamyl transferase
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
NIH	National Institutes of Health
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NTx	N-telopeptide of type I collagen
OC	Osteocalcin
OPG	Osteoprotegerin
PHPT	Primary hyperparathyroidism
PI	Protease inhibitor
PTH	Parathyroid hormone
PYD	Pyridinoline
RANK	Receptor activator of nuclear factor κβ
RANKL	Receptor activator of nuclear factor κβ ligand
RIA	Radioimmunoassay
SD	Standard deviation
SEM	Standard error of the mean
UV	Ultraviolet
WHO	World Health Organisation

# Chapter 1: HIV and Osteoporosis

## Background history of HIV and AIDS:

The first reports of the illness that came to be termed AIDS occurred in 1981 when two clusters of very rare illnesses (*Pneumocystis carinii* pneumonia and Kaposi's sarcoma) occurred in young homosexual men in the United States. In 1983, HIV was identified and proved to be the cause of AIDS. Two years later, an ELISA antibody assay was developed for HIV, allowing accurate diagnosis to occur. In the late 1980s, the first of the antiretroviral drugs, zidovudine, was introduced as monotherapy for treatment of HIV/AIDS. Zidovudine is a NRTI. The use of zidovudine was followed by the development of other NRTIs which were sequentially introduced throughout the 1990s. Currently there are at least eight NRTIs available including zidovudine, didanosine, stavudine, zalcitabine, abacavir, emtricitabine, lamivudine, and tenofovir. In the mid to late 1990s, two new classes of agents were introduced— firstly the PIs followed by the NNRTIs. There are now at least nine PIs available – including tipranavir, fosamprenavir, atazanavir, indinavir, saquinavir, nelfinavir, ritonavir, amprenavir, and lopinavir— and at least three NNRTIs available— including efavirenz, delavirdine, and nevirapine.

Treatment was initially based around monotherapy with zidovudine or didanosine. Later dual therapy with two NRTIs was introduced. In the mid 1990s, trials showed that combination treatment with two NRTIs and a PI leads to near-complete suppression of viral replication. This combination of treatment was termed Highly Active Antiretroviral Therapy or HAART. In 1997, as a result of these trials, the United States guidelines for treatment of HIV/AIDS were changed and HAART was recommended as first line antiretroviral therapy for HIV [11]. While initially HAART referred to the combination of two NRTIs and a PI, later it evolved to mean any combination of at least three agents which causes prolonged viral suppression. The change to the use of HAART as first line treatment has produced substantial improvements in the average life expectancy of HIV-infected patients [12, 13]. HAART has also resulted in improved quality of life, fewer hospital admissions and a lower incidence of malnutrition [14-16]. The longer life expectancy of HAART-treated HIV-infected patients, together with an increasing number of infected patients worldwide, means that the burden of HIV disease is set to increase for the foreseeable future [17]. Thus, it is likely that diseases associated with HIV



infection and adverse effects of antiretroviral treatment will assume greater importance in the management of HIV infection.

### **Osteoporosis prior to the use of HAART:**

Patients infected with HIV are potentially at risk of osteoporosis because of chronic illness, low body weight, hypogonadism, and the use of HAART. The first report suggesting an association between HIV and metabolic bone disease occurred in 1993. In comparison to a control group, 16 HIV-infected patients had lower levels of biochemical markers of bone turnover and BMD that did not reach statistical significance [18]. This report was followed by three other small studies which are summarised in Table 1.1. In a study of 22 HIV-infected patients, markers of bone turnover and BMC were normal although serum OC levels fell with severity of illness. All of the patients underwent a bone biopsy, which demonstrated lower histomorphometric parameters of bone formation and turnover, features which worsened with severity of illness [19]. A larger study of 45 HIV-infected men reported that there was a reduction in BMD at the lumbar spine, femoral neck, and total body when compared to predicted normal values, although only the difference in BMD at the spine reached statistical significance [20]. 21 men had a follow up BMD measurement a mean of 15 months later. There was a 1.0-1.6% reduction in BMD at all sites. The authors felt that this was not excessive, but their interpretation is hindered by the lack of a control group. A small, retrospective study of 23 HIV-infected men and women following the initiation of treatment with two NRTIs showed normal lumbar spine BMD at baseline [21]. Over a mean duration of 22 months of follow-up there was a 4% decrease in BMD. This loss of BMD was accompanied by a 4 kg decrease in body weight that was predominantly loss of fat mass. Together, these four studies suggested that HIV infection may impact upon BMD, however definitive conclusions are not possible because of the small size of the study groups and, in three of the studies, the lack of a control group.

Recently two further studies by the same group were published in which the data was collected prior to 1995 but not published until several years later. The first study reported BMC of the left distal forearm from 100 HIV-infected patients, 30 intravenous drug users, and 40 healthy controls [22]. In women, BMC was lower in HIV-infected women who were not intravenous drug users compared with intravenous drug users (whether HIV-infected or HIV-negative) and the controls. In men, BMC was lower in the HIV-infected groups than the controls regardless of intravenous drug user status. Interpretation of the results is limited by

marked differences in the baseline characteristics between the groups (age, body weight, and, in the women, frequency of amenorrhoea). The second study compared HIV-infected women with healthy controls [23]. Absolute BMD data were not presented– instead percentages of osteopenia and osteoporosis at the lumbar spine and Ward’s triangle were reported. There were increased rates of low BMD at both sites (Table 1.1). However, interpretation of the results of the study is limited by the differences in body weight and frequency of amenorrhoea between the HIV cohort and the control group. Thus, in both studies, the differences in rates of low BMD and BMC between the groups may be attributable to the differences in risk factors for osteoporosis between the HIV groups and the control groups, rather than a direct effect of HIV infection.

## **Osteoporosis in the HAART era:**

### ***Cross-sectional studies:***

Despite a dramatic improvement in the general health status of HIV-infected patients due to treatment with HAART, evidence is beginning to accumulate that chronic HIV infection may be accompanied by important co-morbidities. In recent years, evidence from cross-sectional studies has suggested that HIV-infected patients taking HAART have an increased prevalence of osteopenia and osteoporosis. Osteopenia was first reported as a possible complication of HAART in 2000 by a group from Missouri [5]. 95 HIV-infected men underwent DXA scanning of the whole body and BMD of the lumbar spine, proximal femur and total body were derived from that scan. Those taking a PI had reduced BMD at the lumbar spine and proximal femur compared to those whose treatment regimen did not contain a PI or had not received antiretroviral agents, and 17 HIV-negative controls. 50% of the PI-treated group had osteopenia or osteoporosis at the lumbar spine in comparison to 23% of group who had not received PIs and 29% of the controls. This initial report has been followed by a number of other cross-sectional studies which are summarised briefly here in chronological order (Table 1.2).

Lawal et al. reported on a group of malnourished, HIV-infected men studied prior to the use of HAART, and a group of HIV-infected patients treated with HAART who had fat redistribution and were studied some years later [24]. There was no difference in total body BMD between the HIV-infected groups, but each of the HIV-infected groups had lower total

**Table 1.1: Cross-sectional studies of BMD in HIV-infected patients prior to use of HAART**

Reference	Place, year of publication	Cases						Controls					
		N M/F	Age (Yrs)	Weight/BMI (kg/kgm <sup>-2</sup> )	BMD (g/cm <sup>2</sup> )	Osteopenia %	Osteoporosis %	N M/F	Age (Yrs)	Weight/BMI (kg/kgm <sup>-2</sup> )	BMD (g/cm <sup>2</sup> )	Osteopenia %	Osteoporosis %
[18]	Granada, 1993	16 11/5	29.7	62.4	FN 0.843 L4 1.054			27 19/8	N/A	67.5	FN 0.854 L4 1.106		
[19]	Barcelona, 1995	22 13/9	27.9	N/A	N/A			From manufacturer's database					
[20]	London, 1997	45 45/0	36	21.5	LS 1.165 FN 1.016 TB 1.165	N/A	0	From manufacturer's database					
[21]	Athens, 2002	23 17/6	37.4	24.7	LS 1.053	N/A	N/A	From manufacturer's database					
[23]	Giessen, 2003	50 0/50	37.4	25.1	N/A	LS 62 WT 22	LS 14 WT 0	50 0/50	35.1	26.9	N/A	LS 0 WT 0	LS 4 WT 0

Abbreviations: M male; F female; N/A not available; FN femoral neck; L4 lumbar vertebra 4; LS lumbar spine; TB total body; WT ward's triangle

body BMD compared to age- and sex-matched control groups. However, none of the groups were comparable with respect to weight, lean mass, or fat mass.

Carr et al. reported BMD data from a cohort of HIV-infected men who took part in a study of lipodystrophy prevalence [25]. As a group the total body BMD was normal. However, there were increased rates of osteopenia and osteoporosis (determined from total body BMD) compared to the expected rates for the population. Total body BMD was lower in men receiving HAART compared to HAART-naïve men, but not specifically related to PI therapy. Parameters independently associated with low total body BMD were age, lean body mass and stavudine treatment, while parameters independently associated with osteopenia or osteoporosis at the total body site were serum lactate and low body weight prior to commencement of antiretroviral treatment.

Knobel et al. reported that a cohort of HIV-infected patients had reduced BMD compared to controls at the lumbar spine and at the femoral neck [26]. There were no differences in BMD in HIV-infected patients related to medication use. There were high rates of osteopenia and osteoporosis in both the HIV-infected cohort and the control group that may be in part related to the relatively low BMI for both groups.

Fairfield et al. compared a group of men with AIDS wasting to a control group [27]. BMD at the hip and spine was reduced in the HIV group and was related to weight but not to any HIV-related parameters. There were higher rates of osteopenia and osteoporosis in the HIV group. The control group was on average 14 kg heavier than the HIV group but, after adjustment for this weight difference, AIDS wasting remained a significant association with low BMD. The same group studied a cohort of HIV-infected men and a group of age- and BMI-matched controls to determine the influence of lipodystrophy on BMD [28]. Lumbar spine BMD, as measured by quantitative computed tomography, was reduced in the HIV-infected subgroup with lipodystrophy, but not in the subgroup without lipodystrophy, compared to controls. There was no difference in total body BMD, measured using DXA, between the groups. Using multivariate analysis, only abdominal visceral fat mass independently predicted lumbar spine BMD.

Nolan et al. reported BMD data from HIV-infected men enrolled in the West Australian cohort study [29]. The cohort was split into patients with PI exposure and patients who were PI-naïve. Mean lumbar spine BMD z scores (derived from whole body scans) were lower in

the PI-exposed group compared to the PI-naïve group. In both groups, the mean z score was significantly lower than the expected value of zero. In addition, the PI-exposed group had higher rates of osteoporosis than the PI-naïve group but the rates of osteopenia were similar. Lowest BMI prior to study entry was an independent predictor of BMD.

Complementing the earlier study on AIDS wasting in men, Huang et al. compared a group of women with AIDS wasting and relative androgen deficiency to a control group [30]. BMD was reduced at the lumbar spine, femoral neck, total hip and total body in women with AIDS wasting compared to BMI-matched healthy controls. In addition, there were increased rates of osteopenia but not osteoporosis in women with AIDS wasting. Using multiple regression analysis, lean muscle mass but not HIV-related parameters predicted BMD.

Moore et al. reported high prevalence of osteopenia and osteoporosis in a cohort of HIV-infected men [31]. BMD was lower than expected at the lumbar spine, total hip, and femoral neck. There was no association between any HIV-related parameters or treatment and low BMD.

Similar findings were reported by Gold et al. in a cohort of HIV-infected men who had reduced BMD at the lumbar spine and proximal femur [32]. Using multivariate analysis, reduced BMD was related to age, lean body mass and length of NRTI treatment. Interpretation of the study is limited by the lack of body weight data.

To determine what factors influence the prevalence of osteopenia, Loiseau-Peres et al. compared a group of HIV-infected patients with an age- and sex-matched control group [33]. BMD at the total hip and the lumbar spine was reduced in the HIV-infected men and women compared to the controls. In the HIV-infected group, there was increased prevalence of osteopenia and osteoporosis in males but only increased prevalence of osteopenia in females. Interestingly, BMD was higher in the HIV-infected women than the HIV-infected men at the spine and comparable at the hip. Interpretation of the results is limited by the small size of the study, and the absence of age and body weight data for the groups.

Mondy et al. reported BMD data from HIV-infected people enrolled in a longitudinal study of BMD [34]. There was a higher than expected prevalence of osteopenia or osteoporosis. Low BMD was associated with smoking exposure, steroid use, body weight and history of weight loss, but not with HIV-related parameters or markers of bone turnover.

Vescini et al. reported high rates of osteopenia and osteoporosis at the lumbar spine and total hip in a cohort of HIV-infected patients [35]. Males had higher rates of osteoporosis but not osteopenia compared to females. There was no relationship between BMD and any HIV-related parameter.

Bruera et al. reported that a cohort of HIV-infected people had low BMD compared to healthy controls [36]. The HIV-infected cohort was divided into three subgroups: antiretroviral naïve, HAART including PI, and HAART without PI. There were no differences in BMD at the total body, femoral neck, or lumbar spine between these subgroups, but, at all sites, the BMD of the HIV-infected subgroups was less than the control group. Duration of HIV infection correlated with BMD at all sites. Interpretation of results is limited by a 6-7 kg weight difference between the control group and each of the HIV subgroups.

To determine the effect of HAART and PI treatment on BMD, Fernandez-Riviera et al. reported data from a cohort of HIV-infected patients [37]. BMD at the femoral neck and lumbar spine was lower in those receiving HAART including a PI compared to the subgroup receiving HAART without a PI and the subgroup who had never received HAART. Prevalence of osteopenia but not osteoporosis was increased in those receiving HAART compared to the other subgroups. The presence of osteopenia was related to male sex and to the use of PIs, but not the duration of exposure to PIs. In a later report, the authors found no relationship between osteopenia and levels of free testosterone or vitamin D metabolites [38].

Using BMD data from a cohort of HIV-infected females and an age- and sex-matched control group, Dolan et al. focused on BMD in HIV-infected women [39]. BMD was lower at the lumbar spine, total hip, and femoral neck in the HIV-infected women than the controls. The rates of osteopenia and osteoporosis in the HIV-infected women were approximately double those in the controls. BMD was related to age, body weight and composition, and urine NTx, but not HIV-related parameters. In a later report of a slightly larger cohort of women with almost identical baseline data [40], the authors reported similar findings, although, in this report, duration of NRTI and NNRTI use were predictors of lumbar spine BMD, and duration of HIV infection, CD4 count, and viral load were predictors of total hip BMD.

To determine the effect of treatment on BMD, Amiel et al. compared BMD results from a group of HIV-infected men with a group of age-matched healthy controls [41]. BMD was lower at the femoral neck and lumbar spine and rates of osteopenia and osteoporosis were

higher in the HIV-infected group than the controls. To determine the effects of medication on BMD, the cohort was divided into 3 groups: HAART including PI, HAART without PI, and antiretroviral naïve. There was no difference in BMD between the three groups, but each of the groups had significantly lower BMD than the controls. On multivariate analysis, BMI was a significant predictor of BMD, but BMD was not related to any HIV-related parameters.

Brown et al. reported BMD data from an HIV-infected cohort and control group in a study of the effect of body composition on BMD [42]. The HIV-infected cohort had double the rate of osteopenia or osteoporosis compared with the controls, and BMD was reduced at the hip and spine but not the forearm in the HIV cohort. Low BMD was related to increased central adiposity, and plasma glucose levels after a glucose tolerance test.

Maddedu et al. reported BMD from a cohort of HIV-infected patients and healthy controls [43]. The HIV-infected cohort was divided into three groups: antiretroviral naïve; HAART involving PI, and HAART without PI. All three groups had increased rates of osteopenia at the lumbar spine and proximal femur compared to controls, whereas rates of osteoporosis were increased in those taking HAART compared to both antiretroviral-naïve patients and controls in whom osteoporosis was not detected. Interpretation of the results is limited by the lack of age or body weight data for the control group.

Landonio et al. reported higher than expected rates of osteopenia but not osteoporosis from ultrasound measurements of BMD at the heel in a cohort of HIV-infected patients [44]. The presence of osteopenia or osteoporosis was not related to duration of treatment with HAART or protease inhibitors.

Koutkia et al. enrolled 31 HIV-infected men with central fat accumulation into an intervention trial discussed in more detail later [45]. L4 vertebral BMD was assessed by computed tomography and was lower than expected.

Seminari et al. reported that a cohort of HIV-infected patients had higher than expected rates of osteopenia and osteoporosis [46]. Independent predictors of the presence of osteopenia or osteoporosis were age, and BMI, but not HIV-related parameters.

Bonnet et al. reported that a cohort of HIV-infected men had lower total body BMD than a large control group [47]. BMD was related to the presence of lipodystrophy and treatment with HAART.

Konishi et al. reported a higher than expected prevalence of osteopenia in a cohort of HIV-infected Japanese men [48]. Serum RANKL levels were inversely correlated with lumbar spine BMD and were significantly higher in HAART-treated patients compared to non-HAART-treated or untreated patients

Yin et al. reported BMD data from a cohort of Hispanic and African American postmenopausal women compared with age- and race-matched controls [49]. BMD was significantly lower in the HIV-infected women than the controls at the lumbar spine and total hip. There were increased rates of osteoporosis but not osteopenia in the HIV-infected women compared to the controls. Independent predictors of BMD were lowest historical body weight and years since menopause but not any HIV-related parameters. Interpretation of the findings is limited by a 9 kg difference in body weight between the groups.

Ozçakar et al. reported high prevalence of osteopenia and osteoporosis in a small cohort of HIV-infected patients [50]. BMD at the spine and hip correlated with current CD4 counts. Interpretation of the results is limited by the lack of body weight data and the small size of the cohort.

To determine the effect of ethnicity on prevalence of osteopenia in HIV-infected patients, Curtis et al. compared BMD data from a cohort of African-Americans to a cohort of predominantly (95%) white Americans [51]. There were no between-groups differences in BMD or prevalence of osteopenia and osteoporosis, although for both groups the mean BMD z score was significantly less than zero. Interestingly, BMD in women was higher than men at the spine and femoral neck. Interpretation of the results is limited by the difference in characteristics between the groups (BMI difference of 6 kg/m<sup>2</sup>, and differences in the rates of use of hormone replacement therapy, and anabolic steroids).

Fausto et al. [52-54] reported on potential predictive factors for osteoporosis in a cohort of HIV-infected patients. Female gender, older age, low BMI, and viral load were associated with lower BMD, whereas the use of HAART was not a predictor of osteopenia or osteoporosis [54]. However, in other reports on a similar cohort of patients, duration of HAART was an independent predictor of osteopenia or osteoporosis [52] whereas use of NNRTIs was not associated with low BMD [53].

Arnsten et al. reported BMD data from a large cohort of women with similar behavioural risk factors for HIV infection, approximately 50% of whom were HIV-infected [55]. BMD was



significantly lower in the HIV-infected women compared to the controls. BMD was independently associated with age, race, weight, use of prednisone, use of oestrogen, previous fracture and presence of HIV infection.

Garcia Aparicio et al. reported similar but higher than expected rates of osteopenia and osteoporosis in two small cohorts of HIV-infected men– one cohort of untreated patients, and one cohort of patients treated with HAART that included a PI [56].

### ***Studies where BMC was reported:***

McDermott et al. reported on 203 men and 62 women from Boston who were infected with HIV [57]. BMC in total body and regional DXA scans was reduced in men, but not women, taking HAART. This effect became more pronounced with longer duration of therapy.

Rosenthal et al. reported on the effects of fat mass on regional BMC and BMD in 102 HIV-infected men from Montreal [58]. Men in the quartile with lowest fat mass had lower regional BMC and lower BMD T scores at the total hip than the remainder of the cohort.

### ***Associations between BMD and other variables:***

While BMD has been consistently reported as lower than expected in HIV-infected patients, any further associations are controversial. Although many studies have reported relationships between BMD and other variables, these analyses have been limited by the small size of the cohorts, and the lack of comparable control groups. Many studies have focused on the relationship between HIV-related parameters, antiretroviral treatment, and BMD in an attempt to explain the observed high rates of osteopenia and osteoporosis. The results from these studies have not been consistent. Five studies [5, 29, 31, 37, 43] reported low BMD in those using a PI, but this was not confirmed in later and larger studies [25-28, 30, 32-36, 39, 41, 42, 44, 49, 55, 56]. Other HIV-related parameters that have been inconsistently associated with low BMD include use or duration of HAART [25, 43, 47, 52], duration of NRTI [25, 32, 40], use of NNRTI [28], stavudine treatment [25, 37], lipodystrophy or fat redistribution [28, 42, 47], serum lactate [25], and duration of HIV infection [25, 32, 34, 36, 40]. However, for each of these factors other researchers have reported no relationship with BMD– no relationship was observed between BMD and lipodystrophy [33], use or duration of NRTI [28, 30, 39], use or duration of NNRTI [39, 53], use or duration of HAART [36, 37, 41, 44, 49, 54, 55], serum lactate [39], or duration of HIV infection [31, 41, 49, 55]. Caution needs to be

**Table 1.2: Cross-sectional studies of BMD in HIV-infected adults in the HAART era**

Reference	Place, year of publication	Cases							Controls					
		N (M/F)	Group	Age (Yrs)	Weight/ BMI (kg/kgm <sup>-2</sup> )	BMD (g/cm <sup>2</sup> )	Osteopenia %	Osteoporosis %	N M/F	Age (Yrs)	Weight/ BMI (kg/kgm <sup>-2</sup> )	BMD (g/cm <sup>2</sup> )	Osteopenia %	Osteoporosis %
[5]	St Louis, 2000	95 95/0	+PI 60 -PI 35	41 37	24 22	LS <sup>a</sup> 0.986 LS <sup>a</sup> 1.069	50 23		17 17/0	33	23	LS <sup>a</sup> 1.066	29	
[24]	New York, 2001	58 55/3	PreH 36 PostH 22	N/A	64.2 79.2	TB 1.18 TB 1.17			N/A	N/A	81.9 85.4	TB 1.17 TB 1.23		
[25]	Sydney, 2001	221 221/0		43	73.6	TB 1.22	TB 20	TB 3	From manufacturer's database					
[26]	Barcelona, 2001	80 58/22	+PI 37 -PI 37 -D 26	44 42 36	23 23 22	LS 0.956 <sup>b</sup> LS 0.968 <sup>b</sup> LS 1.044 <sup>b</sup>	70 76 58	30 18 11	100 N/A	40	23	LS 1.064 <sup>b</sup>	25	5
[27]	Boston, 2001	50 50/0		38.1	22.1	LS 1.021 TH 0.951	33		35 35/0	35.5	25.9	LS 1.084 TH 1.070	N/A	N/A
[28]	Boston, 2001	41 41/0	+LD 21 -LD 20	44 41	24.7 24.6	TB 1.09 TB 1.14			18 18/0	42	24.6	TB 1.13		
[29]	Perth, 2001	183 183/0	+PI 131 -PI 52	N/A	N/A	LS z=-0.97 LS z=-0.61	38.2 40	18.3 7.7	From manufacturer's database					
[30]	Boston, 2001	28 0/28		37	21.5	LS 0.94 TH 0.88 TB 1.04	50 48.1	3.6 0	21 0/21	36	22.5	LS 1.03 TH 0.94 TB 1.10	14 9.5	4.8 0
[31]	London, 2001	105 105/0		40	24.4	LS T=-1.36 <sup>b</sup> TH T=-0.53 <sup>b</sup>	58 <sup>c</sup>	13 <sup>c</sup>	From manufacturer's database					
[32]	Sydney, 2002	110 110/0		44	N/A	LS z=-0.3 <sup>b</sup> TH z=-0.3 <sup>b</sup>	44.5 <sup>c</sup>	10.9 <sup>c</sup>	From manufacturer's database					
[33]	Orleans, 2002	47 31/16		M 43 F 39	N/A N/A	LS 0.948 TH 0.919 LS 0.989 TH 0.912	61.3 <sup>c</sup> 56.2 <sup>c</sup>	12.9 <sup>c</sup> 0 <sup>c</sup>	47 31/16	N/A	N/A	LS 1.043 TH 1.010 LS 1.080 TH 0.968	45.2 <sup>c</sup> 6.2 <sup>c</sup>	3.2 <sup>c</sup> 0 <sup>c</sup>
[34]	St Louis, 2003	125 108/17		41	25	LS T=-0.92 <sup>b</sup> TH T=-0.95 <sup>b</sup>	46 <sup>c</sup>		From manufacturer's database					
[35]	Bologna, 2003	70 34/36		41	N/A	N/A	LS 40 TH 46	LS 8.6 TH 10	From manufacturer's database					
[36]	Cordoba, 2003	111 89/22	+PI 34/8  -PI 25/11  -D 30/3	36.3  34.8  31.1	23.8  23.9  23.8	LS 0.97 FN 0.80 TB 1.11 LS 0.97 FN 0.77 TB 1.11 LS 0.97 FN 0.85 TB 1.10	38.5 51.3  44.4 59.3  60.9 65.2	7.7 18.0  7.4 22.2  4.35 4.35	31 24/7	31.4	25.4	LS 1.04 FN 0.91 TB 1.16	36.5 15.4	5.2 0
[37]	Seville, 2003	89 71/18	+PI 63  -PI 15  -D11	37	24.1	LS 0.963 FN 0.808 LS 1.002 FN 0.851 LS 1.002 FN 0.889	LS 30 <sup>c</sup> FN 29 <sup>c</sup>	LS 1 <sup>c</sup> FN 0 <sup>c</sup>	From manufacturer's database					

Reference	Place, year of publication	Cases							Controls					
		N (M/F)	Group	Age (Yrs)	Weight/BMI (kg/kgm <sup>-2</sup> )	BMD (g/cm <sup>2</sup> )	Osteopenia %	Osteoporosis %	N M/F	Age (Yrs)	Weight/BMI (kg/kgm <sup>-2</sup> )	BMD (g/cm <sup>2</sup> )	Osteopenia %	Osteoporosis %
[39]	Boston, 2004	84 0/84		41	26	LS 1.02 TH 0.93	54 <sup>c</sup>	10 <sup>c</sup>	63 0/63	41	27	LS 1.07 TH 0.99	30 <sup>c</sup>	5 <sup>c</sup>
[41]	Paris, 2004	148 148/0		40	23	LS 1.07 FN 0.92	66 <sup>c</sup>	16 <sup>c</sup>	81 81/0	39	24	LS 1.20 FN 1.01	32 <sup>c</sup>	4 <sup>c</sup>
[42]	Washington DC, 2004	51 44/7		40.1	25.2	LS T=-0.84 TH T=-0.31	55 <sup>c</sup>	8 <sup>c</sup>	22 18/4	39.2	25.7	LS T=-0.09 TH T=0.26	32 <sup>c</sup>	0 <sup>c</sup>
[43]	Sassari, 2004	172 112/60	+PI 60/32 -PI 38/22 -D 14/6	39 38 38	22.5 22.6 21.7	LS 0.92 TH 0.84 LS 0.97 TH 0.88 LS T=-0.91 TH T=-0.96	37 40 38 35 30 30	21.7 15.2 8.3 5 0 0	64 39/25	N/A	N/A	LS 0.99 TH 0.89	8 8	0 0
[44]	Milan, 2004	59 44/15	H 32/12 -D 12/3	41.5 37.8	N/A	N/A	32 33	2.3 6.6	From manufacturer's database					
[45]	Boston, 2005	31 31/0		46	26.2	LS z=-0.70			From manufacturer's database					
[46]	Milan, 2005	68 55/13		41 <sup>b</sup>	23.1	N/A	28	13	From manufacturer's database					
[47]	Toulouse, 2005	162 162/0		39	23.3	TB 1.15			241 241/0	39	25.4	TB 1.23		
[48]	Nara, 2005	39 39/0		41.4	N/A	LS 1.079	23	2.6	From manufacturer's database					
[49]	New York, 2005	31 0/31		56	26	LS 0.85 TH 0.83	36 36	42 10	186 0/186	57	28	LS 0.94 TH 0.91	33 29	23 1
[50]	Ankara, 2005	27 15/12		39.5	N/A	N/A	51	33	From manufacturer's database					
[51]	Alabama, 2006	44 29/15		45	28.1	M LS 1.20 F LS 1.30 M FN 0.98 F FN 1.05	42 <sup>c</sup>		From manufacturer's database					
[54]	Milan, 2006	161 103/58	H 74/39 -D 29/19	38.4 39.8	23.1 22.9	LS 0.98 TH 0.92 LS 1.01 TH 0.94	42.5 <sup>c</sup> 35.4 <sup>c</sup>	8.8 <sup>c</sup> 10.4 <sup>c</sup>	From manufacturer's database					
[55]	New York, 2006	263 0/263		44	75	LS 1.21 FN 1.01	21 <sup>c</sup> 12 <sup>c</sup>		232 0/232	45	84	LS 1.24 FN 1.05	15 7	
[56]	Madrid, 2006	30 30/0	+PI 17 -D 13	41 35	22.7 24.1	N/A	53 61.5	12 8	From manufacturer's database					

Table 1.2 Continued

Data are mean or percentage unless stated. <sup>a</sup> Lumbar spine BMD derived from whole body scan. <sup>b</sup> Data are median. <sup>c</sup> Proportion of osteopenia or osteoporosis at any site. Abbreviations: M male; F female; N/A not available; +PI HAART including protease inhibitor; -PI HAART without protease inhibitor; PreH PreHAART; PostH PostHAART; H HAART; -D drug naïve; +LD with lipodystrophy; -LD without Lipodystrophy; FN femoral neck; LS lumbar spine; TB total body; TH total hip; T= T score; z= z score.

exercised in interpreting the correlations and associations reported in these studies. In most cases the hypotheses were not prespecified, the sample sizes were small, and there were multiple statistical tests performed. As none of these HIV-related variables have been consistently associated with low BMD, it is possible that the associations observed were due to a chance effect rather than a biological relationship.

The relationships between BMD and traditional risk factors for osteoporosis have also been reported in many studies. There is greater consistency in these results. Age [25, 28, 32, 38, 39, 46, 52-55], gender [54], time since menopause [49], race [51, 55], smoking [34] and body weight, BMI, fat or lean mass [25-30, 32, 34, 39-42, 46, 49, 52-55, 58] have been associated with low BMD. Several studies reported significant relationships between these traditional risk factors for osteoporosis and BMD while reporting no relationship between BMD and HIV-related parameters [26, 27, 34, 39, 41, 42, 46, 49, 53, 55]. Included in this group of studies were the studies with the fewest methodological flaws, with the largest sample sizes, and with comparable control groups [39, 41, 42, 49, 55]. After adjusting for these traditional risk factors for osteoporosis, HIV remained an independent predictor of BMD in three studies [39, 41, 55] but these analyses were frequently not reported.

### **Summary:**

These studies consistently report reduced BMD in cross-sectional analyses of HIV-infected cohorts with higher than expected rates of osteopenia and osteoporosis. The association has been described in a variety of countries from Europe, North America, South America, and Australia. Although the overwhelming majority of participants have been Caucasian, in those studies where other races have been studied [34, 39, 40, 42, 48-51, 55], there has been no difference in this trend. The association between HIV infection and low BMD appears to be independent of gender, and has also been reported in children [59-67]. Currently there are no published studies which show normal BMD on cross-sectional analysis of a cohort of HIV-infected adults.

Many of these cross-sectional studies have significant methodological weaknesses that may limit the generalisability of their results. A number of the studies do not have a control group [25, 29, 31, 32, 34, 35, 37, 44, 46, 48, 50, 51, 54, 56]. In those studies with control groups, the study group and the control group were often not comparable in terms of age or body weight [5, 24, 27, 36, 41, 49, 55] or did not report these data [24, 33, 43]. Where the groups were not

comparably matched, few studies reported the results of analyses adjusting for the differences between the groups [27, 39, 41, 55]. This raises the possibility that the reported increase in frequency of low BMD in HIV-infected men and women could be explained by the differences between the HIV-infected groups and the control groups (or the people from whom the reference range is derived) other than HIV infection itself. Thus HIV-infected people may have increased risk factors for osteoporosis in comparison to healthy uninfected people.

### ***Longitudinal studies:***

A number of prospective longitudinal studies have now been published which provide more information about bone loss in association with HIV infection.

BMD data from 54 men in the West Australian Cohort study who were treated with HAART and had two bone density scans a mean of 377 days apart were reported by Nolan et al. [29]. All participants received two NRTIs and a PI— either indinavir or nelfinavir. In the nelfinavir-treated group, there was no change in the lumbar spine BMD, while in the indinavir-treated group, the lumbar spine BMD z score increased by 0.31 per year. The changes in BMD over time between the two groups were statistically significant. Factors predicting changes in BMD over time were BMI prior to commencement of HAART and baseline BMD.

One small study reported data from 12 people from South Carolina who were treated with HAART including the PI amprenavir and had an increased BMC of 0.04 kg over 48 weeks [68].

Mondy et al. followed 90 of their original cohort of 125 for 72 weeks [34]. Hip and lumbar spine BMD increased by 2.6% and 2.4% respectively over 72 weeks of follow-up. These changes were statistically significant. The increases in BMD were associated with the magnitude of increase in CD4 count following initiation of HAART, and the presence of an undetectable viral load.

Mallon et al. reported on BMD changes in 40 HIV-infected men with no previous antiretroviral exposure after they commenced HAART [69]. Total body BMD was normal at baseline. The authors reported 'T' scores calculated using spinal BMD derived from the total body scan. The median 'T' score at baseline was 0.1 and it was reported that the 'T' score increased slightly at 24 weeks, and then fell significantly at 48 weeks, remaining lower than

baseline until the end of the study at 144 weeks. The percentage of men with 'T' score less than -1 increased from 13% to 22% over the study. Interpretation of the study results is limited by the lack of data or statistical testing presented in the report.

Fernandez-Riviera et al. followed 70 of their cohort of 89 patients for one year [37]. BMD at the lumbar spine and femoral neck fell by 0.6% and 1.4% respectively; neither result was statistically significant.

Cirreli et al. reported on BMD changes in 40 HIV-infected patients (29 men, 11 women; mean age 39.1 years) from Rome treated with HAART for 12-43 months [70]. BMD decreased by 2% at the lumbar spine over the mean duration of follow-up of 28 months, a result that was not statistically significant.

McComsey et al. reported that BMD remained stable over 48 weeks in 118 HIV-infected patients from the USA, in whom the NRTI stavudine was substituted with either abacavir or zidovudine [71].

Maddedu et al. followed 27 of their cohort of 172 patients for 14 months [43]. BMD decreased by 0.3% at the lumbar spine and 1% at the femoral neck. These changes were not statistically significant.

Martin et al. [72] reported data from a randomised controlled trial where 85 participants taking HAART which included zidovudine or stavudine were randomised to continue their current regimen or change from zidovudine or stavudine to abacavir. After 24 weeks all participants were offered the chance to change to abacavir or continue their previous regimen. Total body BMD in the zidovudine/stavudine group decreased from baseline over a total of 104 weeks of follow-up, whereas there was no change in the abacavir group. The time-weighted difference in BMD between the groups was not statistically significant.

Tebas et al. reported a non-statistically significant increase in BMD of 2% at the lumbar spine in a sub-study of 18 men and women followed for 48 weeks following a change from PI-based HAART to NNRTI-based HAART [73].

Gallant et al. [74] reported BMD data over a three year follow-up period from 601 men and women who were antiretroviral naïve and were commenced on two different HAART regimens. BMD decreased by -1.0% to -2.8% over three years at the lumbar spine or hip, but

appeared to decrease mainly during the first 24-48 weeks and thereafter remain stable or increase.

Dolan et al. performed a two-year prospective longitudinal study of 100 HIV-infected women and 100 controls [40]. There were no differences in the rate of change of BMD at the lumbar spine or hip, however the HIV-infected group had lower BMD throughout the period. Interpretation of the results of this study is complicated by the very high withdrawal rate (75%) in each group during the follow-up period.

### **Summary:**

The 12 longitudinal studies all produced similar results. Although there generally were high levels of osteopenia and osteoporosis at baseline, BMD remained stable over time. However, interpretation of the results of these studies is complicated by the short duration of follow up in these studies, and the lack of control groups. Nevertheless, there appears to be an unexplained discrepancy between the high prevalence of osteopenia and osteoporosis in cross-sectional studies of HIV-infected people taking HAART and the absence of accelerated bone loss over time in these longitudinal studies.

### **Bone turnover markers and calcitropic hormones:**

Several studies have reported on bone turnover markers and calcitropic hormones prior to the introduction of HAART. The results from these studies are shown in Table 1.3. Aukrust et al. from Trondheim, Norway, reported on bone turnover markers in a cohort of HIV-infected patients and a group of age- and sex-matched controls [75]. 16 patients received HAART while 49 patients were treated with dual or monotherapy. OC levels appeared to fall with increasing severity of illness while CTx levels were similar to controls, except in the group of patients with AIDS where they were elevated. The results for OC were consistent with the four pre-HAART studies mentioned previously which reported either decreased levels of OC [18, 22, 23], or decreasing levels with increasing severity of disease [19] in HIV-infected patients. The results for resorption markers are less consistent. One study reported a decreased urine calcium/creatinine ratio [18], another study reported normal levels of hydroxyproline [19], while two studies reported increased urine collagen cross-links [22, 23] in HIV-infected patients. Teichmann et al. reported that serum calcium, 25OHD, 1,25(OH)<sub>2</sub>D, and PTH were lower in HIV-infected cohorts compared to controls [22, 23].

Many studies have reported bone turnover marker data from adult patients treated with HAART. The data from those studies are presented in Table 1.3. Silva Santos et al. from Brazil, reported on bone markers in 69 HIV-infected men and women compared to 50 age- and sex-matched controls [76]. OC levels were reduced in HIV-infected patients compared to controls with the amount of reduction related to the amount of immunosuppression as measured by CD4 counts. A number of studies described previously in the section on cross-sectional studies reported data on bone turnover markers. The results for bone formation markers were inconsistent. OC levels were reported as lowered in two studies, elevated in three studies, and normal in 13 studies (Table 1.3). Similarly, bsALP levels were reported as elevated in one study, decreased in one study, and normal in four studies. Bone resorption markers (CTx, NTx, PYD or DPD) were reported as elevated in approximately 50% of studies, but in the remaining studies they were reported as normal (Table 1.3). Bone turnover markers do not appear to be influenced by the use of PIs [29, 37]. Calcitropic hormones were generally normal in HAART-treated patients (Table 1.3).

Several prospective studies have also reported data on bone turnover markers. Aukrust et al. described the change in bone markers over a two year period following the introduction of HAART [75]. OC levels steadily rose over the 24 months, while serum CTX levels also rose from 6 months to 24 months but the increment did not reach statistical significance. OC levels prior to HAART did not correlate with CTX levels, while during HAART treatment they strongly correlated. The authors suggested HIV infection is associated with a disorder of synchronisation of bone remodelling which is corrected by HAART treatment. In the West Australian cohort study, OC levels increased over time with indinavir but not nelfinavir but remained within the reference range [29]. Mondy et al. [34] reported baseline high levels of OC and borderline elevated bsALP which both remained stable over time. Urine DPD was normal throughout the study but urine PYD was borderline elevated at baseline and remained stable thereafter. Maddedu et al. reported elevated levels of OC, DPD, PYD in subgroups of their cohort at baseline. All markers of bone turnover and calcitropic hormones remained stable during follow-up [43].

### **Summary:**

The results from these studies are inconsistent. The strongest evidence is for elevation of bone resorption markers. Resorption markers, as assessed using several different assays, have frequently been elevated in both cross-sectional and longitudinal studies in adults. However,



**Table 1.3: Studies of HIV-infected adults that report bone turnover markers**

Reference	N M/F	Reference Group	Formation Markers	Resorption Markers	Calcitropic Hormones
<b>Pre HAART</b>					
[18]	16 11/5	CG	↓ OC	↓ Ca/Cr	
[19]	22 13/9	RR	N OC	N OHP	N PTH, N Ca N 25D, N 1,25D
[75]	73 58/15	CG	↓ OC in AIDS N others	↑ CTx in AIDS N others	↓ PTH, ↓ 1,25D in AIDS
[22]	100 65/35	CG	↓ OC	↑ CCL	↓ Ca, ↓ 25D ↓ 1,25D, ↓ PTH
[23]	50 0/50	CG	↓ OC	↑ CCL	↓ Ca, ↓ 25D ↓ 1,25D, ↓ PTH
<b>Post HAART</b>					
[27]	50 50/0	CG	↓ OC	↑ NTx N DPD	
[28]	41 41/0	CG	N OC	N NTx N DPD	N PTH, N 25D
[29]	183 183/0	RR	N OC		
[30]	28 0/28	CG	N OC	↑ NTx	
[34]	125 108/17	RR	↑ OC N bsALP	N DPD N PYD	N PTH, N Ca N 25D, N 1,25D
[36]	111 89/22	CG	N OC	N DPD	N PTH
[37]	81 71/18	RR	N OC	N CTx	
[76]	69 48/21	CG	↓ OC		
[39]	84 0/84	CG	N OC	↑ NTx	N Ca, N PTH N 25D, ↓ 1,25D
[41]	148 148/0	CG	N OC ↓ bsALP	↑ CTx	N 25D N PTH
[42]	51 44/7	CG	N OC	↑ DPD	
[43]	172 112/60	CG	↑ OC ↑ bsALP	↑ DPD	↑ PTH ↓ 1,25D
[46]	68 55/13	RR	N bsALP	↑ DPD	N Ca, N 25D, N PTH
[38]	74 59/15	RR	N OC	N CTx	N Ca, N 25D, N 1,25D N PTH
[49]	31 0/31	RR	N OC N bsALP	N NTx	N Ca, N 25D, N 1,25D N PTH
[50]	27 15/12	RR	N OC N bsALP		N Ca, N 25D, N PTH
[51]	44 29/15	RR	N OC	N NTx	N Ca, N 25D, N PTH
[40]	100 0/100	CG	↑ OC	↑ NTx	N Ca, N 25D
[54]	161 103/58	RR	N OC	↑ NTx	N Ca, N 25D, N 1,25D N PTH

Abbreviations: M male, F female; CG control group, RR reference range; ↑ increased levels in HIV group compared to controls or reference range; ↓ decreased levels in HIV group compared to controls or reference range; N no difference between HIV group and control group or within the normal reference range; Ca/Cr urine calcium creatinine ratio; OHP hydroxyproline; CCL collagen cross links; Ca serum calcium; 25D serum 25-hydroxyvitamin D; 1,25D 1,25-dihydroxyvitamin D.

elevation of resorption markers is not universal. While some studies have shown alteration of bone formation markers, the majority of studies have reported levels within the normal range. Calcitropic hormones were altered in patients with AIDS prior to treatment with HAART, but studies in the HAART era have generally reported normal values. In summary, the results for bone turnover markers are inconclusive, the differences in the results from studies remain unexplained, and it is not possible to draw reliable conclusions on the relationships between bone turnover markers and HIV or HAART.

### **Pathogenesis of bone loss in HIV infection:**

The mechanism of the bone loss observed in the cross-sectional studies is not clear. It may be due to improved patient survival allowing prolonged skeletal exposure to HIV-related factors that induce bone loss, direct skeletal effects of HAART, or both. An alternative explanation is that people infected with HIV have increased exposure to osteoporosis risk factors such as low body weight, hypogonadism and smoking in comparison to healthy people.

Several mechanisms have been proposed to explain how HIV infection may cause abnormalities in bone metabolism and predispose to low BMD. These have been comprehensively summarised in recent reviews [77-95]. Proposed mechanisms include direct infection of cells of the bone and bone marrow microenvironment with HIV, chronic T-cell activation and abnormal cytokine production affecting osteoblast and osteoclast functions, alteration of calcium homeostasis with changes in vitamin D metabolism and PTH function, hormonal changes, opportunistic infections or neoplastic diseases, and adverse effects of drugs. These mechanisms will be briefly outlined here.

HIV has been shown to infect osteoblast-like cell lines [96], although later studies have not detected CD4 expression or HIV in mature osteoblasts [97]. HIV has been identified at autopsy in bone tissue [98, 99]. HIV can also directly infect bone marrow stromal cells, altering the cytokine milieu and thereby causing pancytopenia [100, 101]. In addition, chronic HIV infection leads to chronic T cell activation which induces a proinflammatory cytokine milieu [102]. This proinflammatory cytokine milieu may affect osteogenic cells via the RANKL pathway. RANKL can activate both osteoclasts and osteoclast precursors, and inhibits osteoclast apoptosis. Thus, RANKL activation favours an imbalance of osteoclasts over osteoblasts in the remodelling process [103]. In addition, inflammatory cytokines are capable of inducing osteoclast differentiation and activation independent of the RANKL

pathway [104]. Activated T cells also express RANKL and are therefore capable of inducing the same imbalance [105]. Exposure to HIV or its envelope protein gp120 has been reported to directly induce T cell production of RANKL [106]. A positive feedback loop was reported to exist between RANKL, inflammatory cytokines and HIV replication [107]. Finally, the HIV accessory protein Vpr has been reported to induce RANKL in combination with glucocorticoid receptor stimulation [108]. Thus, a number of mechanisms may lead to increased RANKL mediated osteoclastogenesis in HIV infection.

There are few published clinical data on the RANKL system. In one study, serum RANKL levels were significantly higher in HAART-treated patients than in non-HAART-treated or untreated patients, were inversely proportional to lumbar spine BMD, and were positively correlated with bone turnover markers [48]. In another study serum OPG and RANKL levels were within the normal range although patients with low BMD had higher serum OPG levels than those with normal BMD [46].

HIV infection can cause hypocalcaemia and hypercalcaemia by a number of distinct mechanisms. Hypocalcaemia is commonly associated with abnormalities of Vitamin D metabolism [109, 110]. One study reported normal levels of 25OHD in HIV-infected men and women, whereas levels of 1,25(OH)<sub>2</sub>D were low [111]. The researchers postulated that abnormal 1 $\alpha$ -hydroxylation of 25OHD was the cause of these abnormalities. Levels of 1,25(OH)<sub>2</sub>D also correlated with degree of immunodeficiency and TNF- $\alpha$  levels, but not body weight or history of malabsorption [111]. Other authors have suggested that PIs may inhibit the cytochrome P450 enzymes 25-hydroxylase and 1 $\alpha$ -hydroxylase which mediate conversion of Vitamin D to 25OHD, and 25OHD to 1,25(OH)<sub>2</sub>D [112]. Parathyroid function may also be altered in HIV infection, usually causing hypocalcaemia. Parathyroid tissue can be directly infected by HIV, and parathyroid cells express receptors similar to CD4 which may mediate this infection [113]. Parathyroid infection by opportunistic pathogens such as CMV can also occur with HIV infection [114]. Prior to the HAART era, PTH levels were low in HIV-infected patients in comparison to control patients, with blunted surges in PTH levels in response to EDTA infusions [113, 115]. Hypercalcaemia can also occur in HIV infection, most commonly resulting from granulomatous disease with elevated levels of 1,25(OH)<sub>2</sub>D due to extrarenal 1 $\alpha$ -hydroxylation of 25OHD in granulomata or due to drug therapy [77, 109]. Cytomegalovirus infection can also cause hypercalcaemia which is thought to result from direct osteoclast activation by activated T cells or inflammatory cytokines [116]. Thus,

calcium metabolism can be altered by a variety of disorders in association with HIV infection. However, in most recent studies of patients taking HAART, levels of calcitropic hormones were normal (Table 1.3).

A number of studies have described hormonal abnormalities in HIV-infected patients which may contribute to low BMD. HIV infection may affect the hypothalamic-pituitary-adrenal axis, perhaps due to the effect of chronic illness rather than a specific HIV-related effect. Hypothalamic hypogonadism is common in patients with AIDS, and AIDS wasting disorders, but not uncomplicated HIV infection [117-120]. Increased growth hormone levels and decreased IGF-I levels have been documented in AIDS wasting [120], and decreased corticosteroid levels have been documented in AIDS [117]. Serum IGF-I responses to growth hormone are decreased in men with AIDS-related wasting [121]. All of these hormonal abnormalities potentially may influence bone metabolism, however these studies were all performed prior to the widespread use of HAART, and these hormonal abnormalities may not be as common in people using an effective HAART regimen.

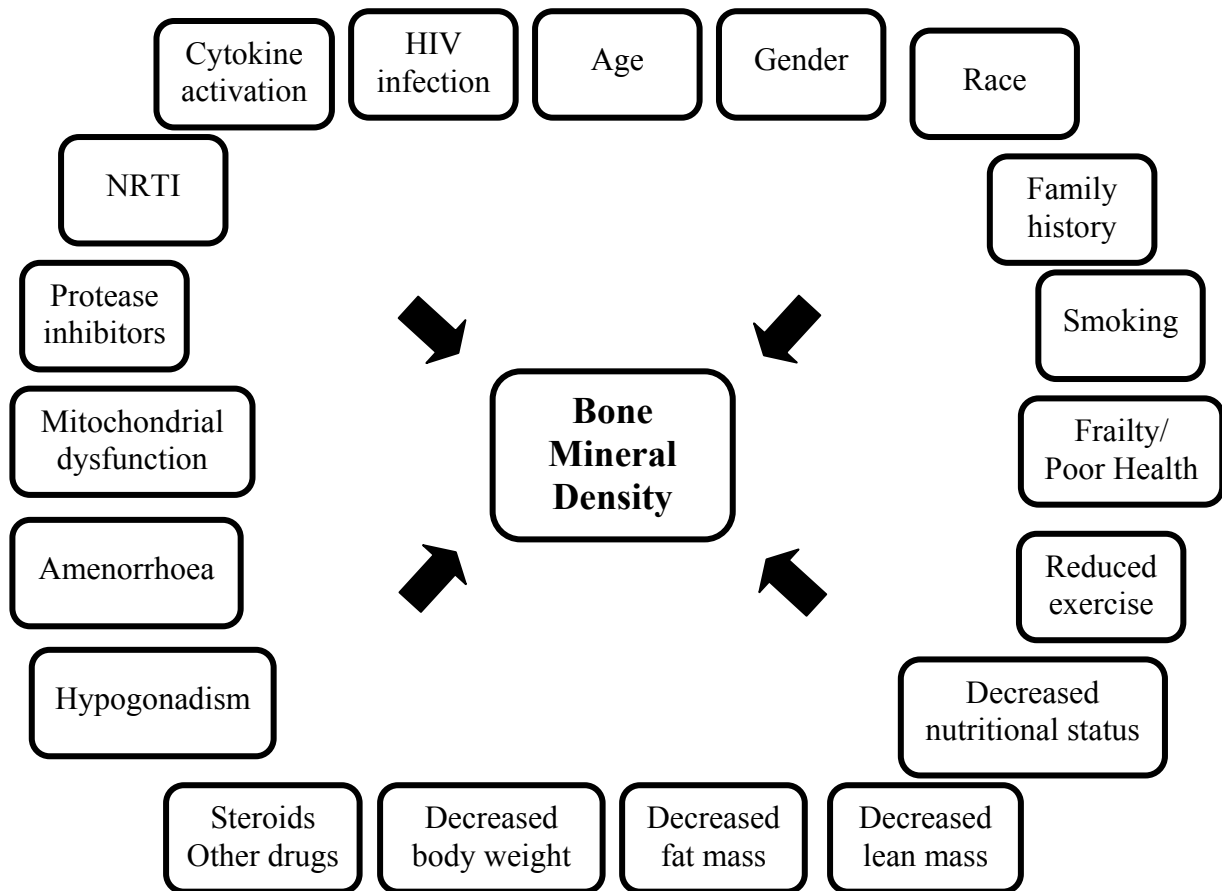
Initial attention focused on the use of protease inhibitors as a potential cause of bone loss following the reports described previously suggesting such an association. *In-vitro*, indinavir has been shown to inhibit osteoblastogenesis, and osteogenesis in cell culture models. In addition, administration of indinavir to mice led to a 12% reduction in spinal BMD [122]. However, another commonly used PI, ritonavir, has the opposite effect on bone [123]. *In-vitro*, ritonavir has been shown to inhibit osteoclast differentiation and reduce bone resorption by interfering with the RANKL signalling pathway. This effect on osteoclastogenesis was also observed in mice exposed to ritonavir [123]. Different responses of bone cells to different PIs have also been found by other groups. Jain and Lenhard observed that indinavir, ritonavir, saquinavir and nelfinavir all increased osteoclast activity in cell culture systems whereas amprenavir and lopinavir had no effect [124]. They also noted that different PIs had different effects on adipocyte differentiation. As osteoblasts and adipocytes have common precursors [125], the authors felt this was further evidence to support the hypothesis that PIs affect osteogenesis. Fakruddin and Laurence reported that ritonavir and saquinavir, but not indinavir or nelfinavir, removed a physiological block preventing RANKL over-activity and increased osteoclastogenesis [106]. Thus, there is evidence from animal and *in-vitro* studies that PIs may affect bones and that effects may be specific to individual agents.

Other authors have speculated that the observed bone loss may be caused by the NRTIs or the combination of NRTIs and PIs as used in HAART regimens. One explanation is that mitochondrial toxicity due to NRTIs, especially stavudine, or PIs may directly affect bone cells, or may cause lactic acidosis which leads to loss of hydroxyapatite from bone [25, 80]. Another explanation is that NRTIs may also affect osteoclastogenesis. Pan et al. reported that treatment with zidovudine led to an increase in osteoclastogenesis in mouse pre-osteoclasts *in-vitro*, and *in-vivo* produced a 10% reduction in mice subcranial BMD with histological features of osteoporosis [126].

Lastly, a number of risk factors for osteoporosis such as decreased physical activity, prolonged bed rest associated with chronic illness, general ill health, malnutrition, low body weight, smoking, and hypogonadism are present in patients with advanced HIV infection [83]. HIV infection may therefore act to promote low BMD through exposure to these traditional risk factors for osteoporosis. However, this hypothesis is not supported by the finding of low BMD in HIV-infected patients in studies following the introduction of HAART treatment where patients are generally of good health.

In summary, the development of low BMD in HIV-infected patients may involve many factors, both traditional osteoporosis risk factors, and novel HIV-related factors. At the present time the exact role of these factors remains to be determined. A current hypothesis of bone loss in association with HIV infection is shown in Figure 1.1.

**Figure 1.1: Factors that may contribute to low BMD in HIV-infection**



**Clinical fractures in HIV infection:**

There are few reported cases of clinical events due to the effects of HIV or HAART on BMD. Stephens et al. reported on two young women infected with HIV who developed osteoporosis [127]. A 29 year old African woman with a history of tuberculosis and secondary amenorrhoea was admitted to hospital with streptococcal pneumonia, tricuspid valve thrombosis, and an acute L2 vertebral fracture. Her BMI was 21.6 kg/m<sup>2</sup>. Lumbar spine BMD T score was -3.2. She had been treated with HAART for two months. A 33 year old African woman with AIDS treated with HAART for 3 years suffered vertebral fractures of T11, L2, and L5. Lumbar spine BMD T score was -3.3. Her BMI was 20.1 kg/m<sup>2</sup> and she had had amenorrhoea for six months with a hormonal profile consistent with premature ovarian failure. The authors considered that the cause of the osteoporosis in both cases was likely to be multifactorial, with important risk factors for osteoporosis present in both cases – ill health, low body weight, presumed vitamin D deficiency, and hypogonadism – but felt that HAART was partially implicated.

Guaraldi et al. reported on fractures in two HIV-infected men with low bone density [128]. A 51 year man sustained a rib fracture following sneezing. Lumbar spine BMD T score was -1.5. A 49 year old man suffered a L1 vertebral fracture following trivial trauma. Lumbar spine T score was -4.8. Both patients had received HAART for 3 years. The authors speculated that the fractures were due to low BMD caused by HAART. Very limited clinical history was presented in this report, but both patients were reported as having had AIDS with significant past illnesses (tuberculosis, *Pneumocystis carinii* pneumonia, and dementia), and had a BMI of 22 and 24 kg/m<sup>2</sup> respectively. As with the previous case, the cause of the low BMD seems likely to be multifactorial rather than specifically due to HIV infection or HAART treatment.

Forsyth et al. reported an unusual fracture in a 50 year old HIV-infected man who had received HAART for several years [129]. He presented with a history of acute pain and swelling in his foot. There was no history of trauma. He failed to respond to treatment with antibiotics for presumed cellulitis and subsequent X-rays of his foot confirmed fractures of the second and third metatarsal. Hip BMD T score was -3.2. He had no other risk factors for osteoporosis.

Panayotakopoulos et al. reported that a 43 year old man who had been infected with HIV for eight years and treated with ritonavir and saquinavir for approximately six months sustained multiple vertebral fractures followed by osteonecrosis of the femoral head [130]. DXA results were not reported but the patient was said to have generalised osteoporosis. He had recently been diagnosed with Burkitt's lymphoma and had received six cycles of chemotherapy that included high dose prednisolone. The authors called for prospective studies and guidelines to aid in the assessment and management of osteoporosis in HIV-infected patients.

McComsey et al. retrospectively assessed records from nine HIV clinics in order to establish the frequency of fragility fractures in community-dwelling HIV-infected patients [131]. 55 fragility fractures were identified from approximately 8600 patients. Only 20% of patients had a measurement of BMD: 36% had osteoporosis at the lumbar spine or hip, and 36-45% had osteopenia at the spine or hip. The nature of the study design did not allow predictive factors for fracture to be determined.

Martin et al. retrospectively surveyed the records of 2700 patients over a 3½ year period for symptomatic bone disorders [132]. Only six cases with BMD T score less than -2 in the

presence of a fragility fracture were identified. All six cases had important traditional risk factors for osteoporosis. There was no difference in the incidence of fragility fractures between PI- or NNRTI-based HAART.

### **Summary:**

Evidence for clinical consequences of the reported high rates of osteopenia and osteoporosis (i.e. increased fracture risk) in HIV-infected cohorts is lacking. Only five cases of fractures have been reported, and in four of those cases important traditional risk factors for osteoporosis were present. In retrospective reviews of HIV-infected clinic patients, the rates of symptomatic osteoporosis and fragility fracture were low. Thus, at this time there is no convincing evidence that there are any clinical consequences relating to low BMD in association with HIV infection. However, this may be in part related to the young age, and therefore low fracture risk, of most HIV-infected patients.

### **Treatment of osteoporosis in HIV infection:**

There have been very few reports of treatment of osteopenia or osteoporosis in HIV-infected patients. Fairfield et al. randomised 54 eugonadal men with AIDS wasting to receive 200 mg intramuscular testosterone weekly or placebo, and an exercise programme of resistance training or no exercise programme in a 2\*2 factorial design [27]. Lumbar spine BMD derived from the total body scan increased from baseline by 2.4% over a 3 month period with testosterone treatment, and the between-groups difference for testosterone versus placebo was 3.7%. Resistance training had no effect on BMD.

Guaraldi et al. described the use of the alendronate 10 mg daily for 6 months in a 51 year old man infected with HIV who suffered a fractured L1 vertebra [133]. BMD improved from lumbar spine T score -3.85 to -2.35 with treatment. There was resolution of clinical symptoms over this time frame.

There are three studies of bisphosphonate treatment in patients infected with HIV. Guaraldi et al. reported a 52 week, randomised, open-label trial of 70 mg weekly alendronate compared to placebo in 41 HIV-infected patients who had been taking HAART for at least 6 months and had a BMD T score less than -1 [134]. All participants took calcium carbonate 1000 mg daily and vitamin D 500 IU daily. Lumbar spine BMD increased by 4% in the alendronate group and 3.7% in the control group, while femoral neck BMD fell by 0.5% in the alendronate



group and 3.5% in the control group. These between-groups differences were not statistically significant.

Mondy et al. reported a 48 week, randomised, open-label trial of 70 mg weekly alendronate compared to placebo in 31 HIV-infected patients treated with HAART for at least 6 months who had a lumbar spine BMD T score less than -1 [135]. All participants received 1000 mg calcium and 400 IU Vitamin D daily. BMD at the lumbar spine increased by 5.2% in the alendronate group compared with 1.3% in the control group ( $P < 0.05$ ), whereas there were no between-groups differences at the femoral neck, trochanter, total hip or total body. Bone turnover markers decreased in both groups but the between-groups difference was only statistically significant for bsALP.

Negredo et al. [136] reported a 96 week, randomised, open-label trial of 70 mg weekly alendronate with dietary counselling to ensure a daily calcium intake of at least 1200 mg compared to dietary counselling alone in 25 HIV-infected patients taking HAART with BMD T score less than -2.5. There were increases in BMD at the lumbar spine and hip in the alendronate group whereas BMD tended to decrease in the control group. The between-groups differences in BMD were only statistically significant at the trochanter.

Koutkia et al. randomised 31 HIV-infected men with central fat accumulation to 1 mg twice daily growth hormone releasing hormone (GHRH) or placebo for 12 weeks [45]. BMD, as assessed by computed tomography of the L4 vertebra, did not change over the 12 weeks with GHRH treatment. There was an increase in bone turnover markers (both formation and resorption) in the GHRH treated group compared to placebo.

### **Summary:**

There are few reliable data for treatment of osteopenia or osteoporosis in HIV-infected patients. The conflicting results among the three trials of bisphosphonate use may be due to the small size of the studies, or the differences in study design and duration of follow up. Overall, more evidence is required before any recommendations can be made about treatment of osteopenia or osteoporosis in association with HIV.

## **Conclusion:**

There is consistent evidence from many cross-sectional studies that people infected with HIV have lower BMD than their unaffected healthy peers or than age- and gender-matched values from reference databases. The cause of the low BMD is uncertain and may be in part due to direct HIV- or HAART-related effects on BMD and in part due to greater exposure to traditional risk factors for osteoporosis in HIV-infected people. There is an unexplained discrepancy between the high prevalence of osteopenia and osteoporosis in HIV-infected people taking HAART in cross-sectional studies and the absence of accelerated bone loss over time in longitudinal studies. In addition, there are surprisingly few reports of clinical skeletal events in HIV-infected people related to low BMD.

The purpose of the subsequent chapters in the first part of this thesis is to compare BMD, both cross-sectionally and longitudinally, in a cohort of HIV-infected men to BMD from a cohort of healthy unaffected controls, and to explore the use of an intravenous bisphosphonate treatment for HIV-infected men with low BMD.

## **Chapter 2: A cross-sectional analysis of bone mineral density in HIV-infected men treated with HAART**

### **Introduction:**

As discussed in Chapter 1, researchers have recently reported low BMD and higher than expected rates of osteoporosis in HIV-infected adults treated with HAART [5, 24-37, 39-56]. The cause of the low BMD remains uncertain and may be due to HIV-related factors, or increased exposure to more traditional risk factors for osteoporosis such as chronic illness, low body weight, smoking and hypogonadism. Many of the previous cross-sectional studies have relied on comparisons of data from HIV-infected patients with densitometer reference databases, or have not controlled for risk factors for osteoporosis which are important potential confounders.

We performed a cross-sectional analysis of data obtained from a cohort of HIV-infected Caucasian men treated with HAART who were aged-matched to a group of healthy, community-dwelling controls. We sought to determine whether BMD in HIV-infected men treated with HAART is different from that of healthy controls, and, if so, what HIV-related factors may explain this finding.

### **Methods:**

#### ***Subjects:***

Between February 2003 and March 2004, all HIV-infected men (approximately 220) who attended infectious disease clinics at our institution were approached by their primary care physician to participate in a clinical study. 72 men agreed to take part. Men were eligible to participate if they had been treated with HAART for at least three months. We excluded men with significant renal, hepatic or thyroid dysfunction; concurrent major systemic illness including malignancy; metabolic bone disease; or current use of a bisphosphonate or systemic glucocorticoids. In addition, we recruited a control group from two sources: 40 healthy men were recruited by workplace advertisements and 222 men aged over 40 years were recruited by newspaper advertisements for a study of calcium supplementation. The exclusion criteria for the HIV-infected men were also applied to the control groups. Other specific exclusion criteria for the controls included any chronic medical condition that required regular systemic

medication; and a history of kidney stones. The study received ethical approval from the Auckland Ethics Committee.

All 72 HIV-infected men were eligible for this cross-sectional analysis, while 173 of 222 and 37 of 40 controls, respectively, were eligible. The 52 potential controls who were excluded had either a chronic medical condition or a history of kidney stones. Each HIV-infected man was age-matched within 5 years to two controls who were randomly selected from the pool of eligible control subjects. Due to the small numbers of eligible non-Caucasian HIV-infected men, and difficulty in finding sufficient comparable controls for them, we restricted our analysis to Caucasian men. Thus we report data from 59 Caucasian HIV-infected men and 118 age-matched healthy Caucasian controls.

### ***Measurements:***

Height was measured at baseline using a Harpenden stadiometer and weight was recorded using electronic scales. All men supplied a fasting blood sample and a fasting second-voided morning urine sample. The following assays were used: serum 25OHD was measured by RIA (DiaSorin, Stillwater, USA); serum PTH and serum testosterone by electrochemiluminescence immunoassays (E170, Roche); serum 1,25(OH)<sub>2</sub>D by RIA (IDS, Boldon, UK); urine NTx by ELISA (Ostex, Seattle, USA). Each of these analytes was measured in the HIV-infected men; serum 25OHD was measured in all controls, while serum PTH, serum testosterone and urine NTx were measured in a subgroup of 34 controls. This subgroup of controls had similar baseline characteristics to the entire control group.

Body composition and BMD of the lumbar spine, proximal femur and total body were measured using a Lunar Expert DXA (GE Lunar, Madison, WI) or a Lunar Prodigy DXA (GE Lunar, Madison, WI). A change in densitometer occurred during the study. All the HIV-infected men and 27 controls had BMD measured using the Expert DXA, while 91 controls had BMD measured using the Prodigy DXA. For this study, data from each scan performed using the Prodigy DXA were converted to predicted Expert DXA values which were then used in the final analyses.

To allow interconversion of BMD data between densitometers, 64 people not involved in the present study, with a variety of indications for BMD measurement, underwent BMD measurements of the lumbar spine, total body and total hip on both machines on the same day. Data from half of this sample were then analysed using linear regression to develop models

allowing the conversion of data from the Prodigy DXA to the Expert DXA. Data from the remaining half of the sample were used to validate the performance of these models in predicting actual data from those not included in the model building phase. Weight, height, BMI, and gender were considered as potential confounders. However, there was no improvement in fit for the models when each of these variables was added to the models alone, or in combination, and therefore these variables were not included in the final equations. The percentage differences (95% CI) between actual data and predicted data were -0.097% (-1.00% to 0.80%) for total body, 0.19% (-1.26% to 1.63%) for L1-L4, and -0.38% (-1.51% to 0.74%) for total hip. The equations generated were: for L1-L4 Expert BMD=  $1.117 * \text{Prodigy BMD} - 0.129$ ; for total body Expert BMD=  $0.966 * \text{Prodigy BMD} - 0.0188$ ; for total hip Expert BMD=  $1.036 * \text{Prodigy BMD} - 0.0318$ .

### ***Statistics:***

Comparisons were made between groups according to HIV status for continuous variables using Student's t-test, and for categorical variables using the Chi-square test. Pearson correlation analysis was used to test for significant linear correlations between variables. Adjustment for potential confounders was performed by analysis of covariance (ANCOVA). Significance level was set at  $P < 0.05$  and all tests were two-tailed. The study had 80% power (at the 5% significance level) to detect a difference of 0.4 SD in BMD between groups at the lumbar spine ( $0.07 \text{ g/cm}^2$ ) or total hip ( $0.06 \text{ g/cm}^2$ ). All statistical analyses were obtained using SPSS for Windows (SPSS Inc., Chicago, IL version 12.0.1) or the SAS software package (SAS Institute, Cary, NC version 9).

### **Results:**

Descriptive and biochemical characteristics of the study groups are summarised in Table 2.1. The HIV-infected group was on average 6.3 kg lighter than the control group. Differences in both fat mass (4.4 kg) and lean mass (2.4 kg) contributed to this weight difference. The HIV-infected group also had increased smoking exposure in the past and at study entry in comparison to the control group.

**Table 2.1: Baseline characteristics of HIV-infected group and control group**

	HIV (N=59)	Controls (N=118)	P
<b>Age</b>	50.1 (8.3)	49.8 (8.7)	0.84
<b>Height (cm)</b>	176.8 (6.1)	177.6 (7.0)	0.44
<b>Weight (kg)</b>	77.1 (10.2)	83.4 (12.8)	0.001
<b>BMI (kg/m<sup>2</sup>)</b>	24.7 (3.0)	26.4 (3.5)	0.002
<b>Fat Mass (kg)</b>	14.5 (7.5)	18.9 (7.8)	0.001
<b>Lean Mass (kg)</b>	57.7 (6.7)	60.1 (7.2)	0.038
<b>Percent Fat</b>	19.4 (7.9)	23.2 (6.8)	0.001
<b>Dietary Calcium (mg/day)</b>	947 (474)	938 (453)	0.92
<b>Previous Smoker</b>	61%	37%	0.002
<b>Current Smoker</b>	29%	4%	< 0.001
<b>Pack-year History</b>	12.1 (14.4)	3.9 (8.2)	< 0.001

Data are mean (SD) or percentage, P for difference between groups.

Baseline clinical characteristics of the HIV-infected group are shown in Table 2.2. The mean duration of known infection was 8.5 years. 90% of participants had homosexually-acquired HIV infection. With advancing severity of HIV infection, patients lost an average of 3.8 kg of body weight (difference between initial and nadir body weights recorded at infectious disease clinic reviews). However by the time of entry to this study, participants had regained this weight and were on average 1 kg heavier than at their first visit to the infectious disease clinic. As expected, CD4 count decreased with advancing HIV infection to a mean nadir of 138 cells/ $\mu$ L, but at the time of entry to this study the mean CD4 count was within the normal range. The mean duration of treatment with any antiretroviral medication was 68 months, and the mean duration of treatment with HAART was 52 months. The median number of times a change had been made to the antiviral medication regimen prior to study inception was two for each patient.

Table 2.3 shows biochemical data, calcitropic hormones, and bone-related laboratory parameters for both groups. Fasting glucose, serum GGT, and serum ALP were significantly higher in the HIV-infected group compared to controls. Albumin-adjusted serum calcium and serum 25OHD levels were significantly lower in the HIV-infected group compared to controls, although the mean levels of both were within the reference range. There were no significant differences between the groups for serum phosphate, serum PTH, serum testosterone, fasting urine calcium and fasting urine NTx. Serum 1,25(OH)<sub>2</sub>D and lactate levels were only measured in the HIV-infected group, and mean values were within the reference range [1,25(OH)<sub>2</sub>D 112 (39) pmol/L, lactate 1.5 (0.7) mmol/L, data are mean (SD)].

**Table 2.2: Clinical characteristics of HIV-infected group**

Time since diagnosis (years)	8.5 (5.2)
Mode of infection- Homosexual	89.8%
Other	10.2%
AIDS defining illness	31%
Lipodystrophy	56%
First recorded body weight (kg) <sup>a</sup>	76.3 (12.4)
Body weight nadir (kg) <sup>a</sup>	72.5 (11.1)
First recorded CD4 count (cells/ $\mu$ L) <sup>a,b</sup>	295 (220)
CD4 count nadir (cells/ $\mu$ L) <sup>a,b</sup>	138 (105)
CD4 count at study entry (cells/ $\mu$ L) <sup>a,b</sup>	563 (222)
First recorded viral load (log copies/mL) <sup>a</sup>	4.4 (0.8)
Viral load peak (log copies/mL) <sup>a</sup>	4.6 (0.7)
Viral load at study entry (log copies/mL) <sup>a</sup>	2.0 (0.8)
<b>Medication – percentage ever used</b>	
NRTI	100%
NNRTI	69%
PI	71%
<b>Medication – duration of treatment</b>	
NRTI (months) <sup>c</sup>	66.1 (40.4)
NNRTI (months) <sup>c</sup>	17.1 (15.7)
PI (months) <sup>c</sup>	35.6 (29.1)
Duration of treatment (months)	67.8 (40.6)
Duration of HAART (months) <sup>d</sup>	52.1 (22.9)
Duration of HAART >12 months	94%
Number of regimen changes <sup>e</sup>	2 (0-10)

Data are mean (SD) or percentage except for number of regimen changes which is expressed as median (range).

<sup>a</sup> All patients were followed regularly at infectious disease clinics after diagnosis of HIV infection was made. Values refer to data extracted from infectious disease clinic notes. Thus first recorded body weight refers to the first ever recorded body weight in the clinic notes.

<sup>b</sup> Reference range: 500-1650 cells/ $\mu$ L

<sup>c</sup> Duration of therapy with NRTI, NNRTI, and PI refer to the number of months that treatment included any agent from the respective class.

<sup>d</sup> HAART was defined as an HIV treatment regimen containing at least three antiretroviral agents.

<sup>e</sup> Number of times any changes were made to the antiretroviral medication regimen.

There were no statistically-significant differences in BMD between groups at either the lumbar spine [HIV-infected group 1.23 (0.18) g/cm<sup>2</sup>, control group 1.25 (0.17) g/cm<sup>2</sup>; P= 0.53] [data are mean (SD)] or total body [HIV-infected group 1.18 (0.09) g/cm<sup>2</sup>, control group 1.20 (0.09) g/cm<sup>2</sup>; P= 0.09]. At the total hip, the HIV-infected group had a small, statistically-significant reduction in BMD compared to the control group [HIV-infected group 1.03 (0.13) g/cm<sup>2</sup>, control group 1.09 (0.14) g/cm<sup>2</sup>; P= 0.01]. Figure 2.1 shows the unadjusted BMD at each site for each participant. 28.8% of the HIV-infected group had osteopenia (BMD T score between -1 and -2.5) at the total hip or lumbar spine compared to 21.2% of the

**Table 2.3: Calcitropic hormones and bone-related laboratory parameters of HIV-infected group and control group**

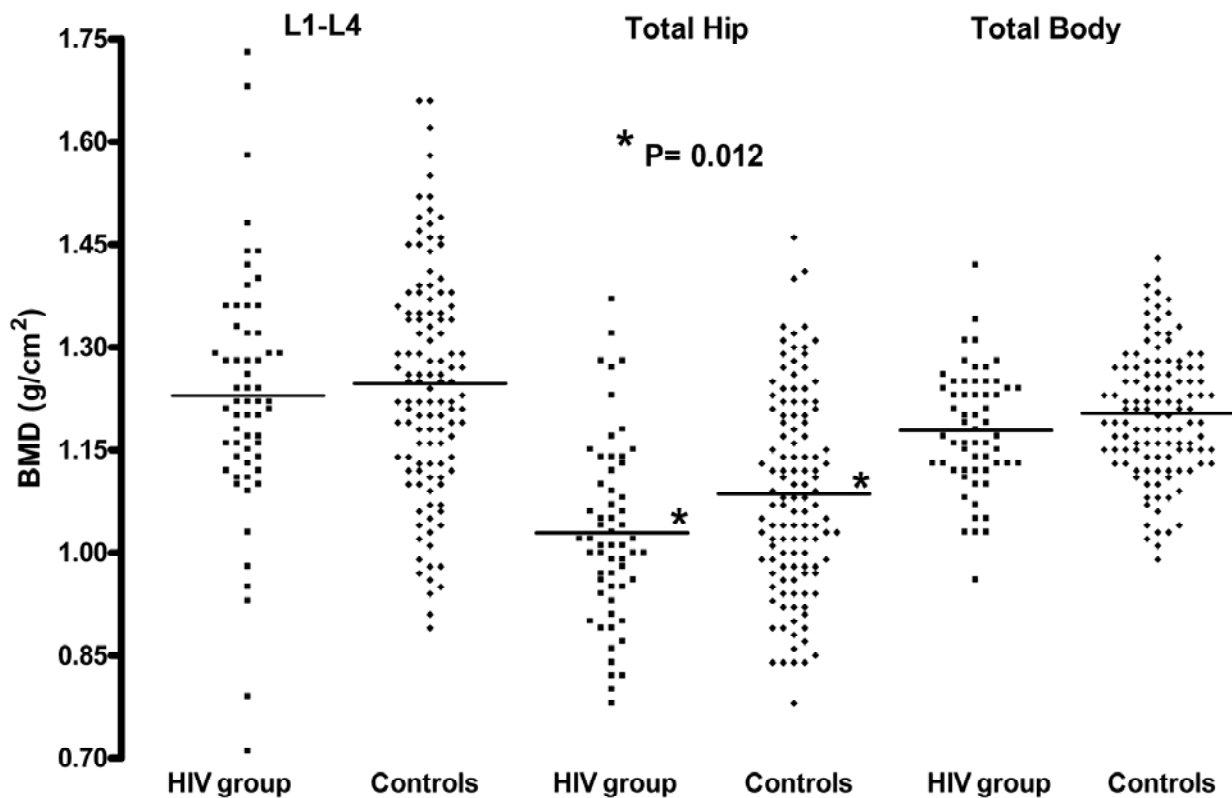
	HIV N=59	Controls N=118	P	Reference Range
<b>Creatinine (mmol/L)</b>	0.08 (0.02)	0.09 (0.01)	0.001	0.05- 0.12
<b>Fasting glucose (mmol/L)</b>	5.8 (2.1)	4.9 (0.4)	< 0.001	3.0-5.6
<b>GGT (U/L)</b>	66 (59)	26 (14)	< 0.001	0-60
<b>Alkaline phosphatase (U/L)</b>	89 (28)	67 (17)	< 0.001	40-120
<b>Albumin (g/L)</b>	42 (4)	44 (2)	< 0.001	35-47
<b>Serum adjusted calcium (mmol/L)</b>	2.26 (0.1)	2.31 (0.1)	0.001	2.10-2.55
<b>Serum phosphate (mmol/L)</b>	1.0 (0.2)	1.0 (0.1)	0.24	0.70-1.50
<b>PTH (pmol/L)</b>	4.2 (1.7)	3.9 (2)	0.49	1.7-7.3
<b>25OHD (nmol/L)</b>	70 (23)	86 (28)	< 0.001	50-150
<b>Testosterone (nmol/L)</b>	18 (9)	17 (5)	0.62	11-35
<b>Urine calcium/creatinine (mmol Ca/mmol Cr)</b>	0.21(0.19)	0.19 (0.13)	0.59	0.06- 0.4
<b>Urine NTx (nmol BCE/mmol Cr)</b>	37 (22)	33 (13)	0.41	3-51

Data are mean (SD), P for difference between groups. Testosterone, PTH, and urine NTx were measured in a subgroup of 34 controls. Urine calcium/creatinine was measured on a fasting 2hr post-voiding sample and its units are mmol calcium / mmol creatinine. Urine NTx was measured on a fasting second morning urine sample and its units are nmol of bone collagen equivalents / mmol of creatinine.

controls (P= 0.27); 3.4% of the HIV-infected group had osteoporosis (BMD T score  $\leq$  -2.5) at the lumbar spine or total hip compared to 0.8% of the controls (P= 0.26). Infection with HIV did not predict the presence of osteopenia or osteoporosis (P= 0.26). The duration of treatment with HAART was not correlated with BMD at any site (r -0.037 to 0.084, P> 0.5 for all 3 sites). As there were differences between the HIV-infected group and the control group in body weight and smoking exposure, both established osteoporosis risk factors, we adjusted for these potential confounders by ANCOVA. Figure 2.2 shows the mean BMD for the HIV-infected group and control group at each site adjusted for the weight difference between groups. The differences in mean BMD between groups after adjusting for the body weight differences were not statistically significant at any site [lumbar spine: HIV-infected group 1.25 ( $\pm$  0.04) g/cm<sup>2</sup>, control group 1.24 ( $\pm$  0.03) g/cm<sup>2</sup>, P= 0.79; total body: HIV-infected group 1.19 ( $\pm$  0.02) g/cm<sup>2</sup>, control group 1.20 ( $\pm$  0.01) g/cm<sup>2</sup>, P= 0.76; total hip: HIV-infected group 1.05 ( $\pm$  0.03) g/cm<sup>2</sup>, control group 1.08 ( $\pm$  0.02) g/cm<sup>2</sup>, P= 0.18] [data are mean ( $\pm$  95% CI)]. Adjusting for smoking exposure in addition to body weight produced similar results.

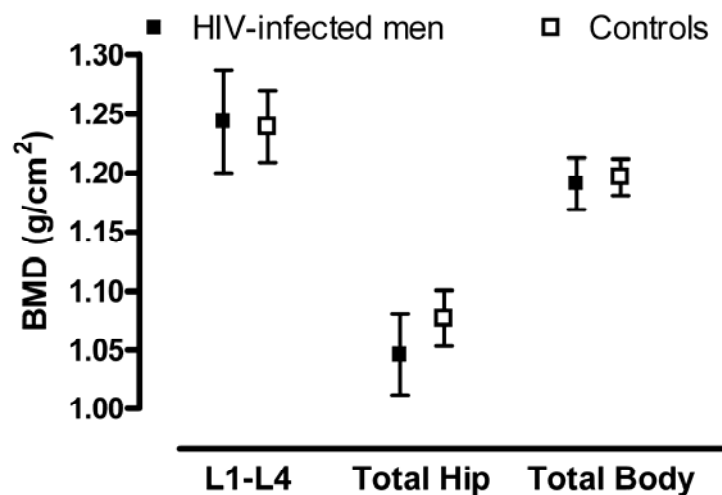


**Figure 2.1: Distribution of unadjusted BMD for HIV-infected group and control group at the lumbar spine, total hip, and total body**



The horizontal lines represent the mean BMD for each group at that site.

**Figure 2.2: Mean BMD and 95% CI at the lumbar spine, total hip, and total body adjusted for body weight in HIV-infected group and controls**



We compared BMD data from the two study groups to age- and gender-matched controls from the Lunar Expert-XL US/Northern Europe database. The mean (SD) z score and P (mean z score= 0) for the groups at each site were as follows– lumbar spine: HIV-infected group 0.26 (1.48), P= 0.18, control group 0.40 (1.37), P= 0.002; total hip: HIV-infected group -0.04 (1.02), P= 0.74, control group 0.39 (1.08), P< 0.001; total body: HIV-infected group -0.46 (1.07), P= 0.002, control group -0.16 (1.12), P= 0.13. Of note is that the mean weight of men in the Lunar Expert-XL US/Northern Europe database was 78 kg, 0.9 kg more than the mean weight of the HIV-infected group and 5.4 kg less than the mean weight of our control group. Thus, when using a control group with comparable body weight to the HIV-infected group there were no statistically significant differences in BMD at the lumbar spine or total hip, but a small reduction in total body BMD. The 5.4 kg weight difference between our control group and the Lunar reference group may in part explain why the z scores of our control group at the total hip and lumbar spine were greater than the expected value of zero.

HIV-related lipodystrophy has previously been associated with low BMD [25, 28, 42]. 33 (56%) of the HIV-infected men in this cohort had evidence of lipodystrophy (evidence of peripheral fat loss or central fat accumulation) on clinical examination. To determine the effect of lipodystrophy on BMD, we compared these 33 men to the 26 men with no lipodystrophy. Baseline characteristics of the HIV-infected group with relation to lipodystrophy are shown in Table 2.4. There were no differences in body weight or smoking exposure between these two groups. Mean BMD at all three sites tended to be lower in the group with lipodystrophy, but the difference was only statistically significant at the total body [lumbar spine: lipodystrophy group 1.20 (0.14) g/cm<sup>2</sup>, non-lipodystrophy group 1.27 (0.22) g/cm<sup>2</sup>, P= 0.15; total body: lipodystrophy group 1.15 (0.06) g/cm<sup>2</sup>, non-lipodystrophy group 1.21 (0.10) g/cm<sup>2</sup>, P= 0.01; total hip: lipodystrophy group 1.00 (0.12) g/cm<sup>2</sup>, non-lipodystrophy group 1.06 (0.15) g/cm<sup>2</sup>, P=0.07; data are mean (SD)]. Adjusting for the differences in age, body weight, fat mass or lean mass between the two groups did not change the results. We also compared the 33 men with lipodystrophy to the control group. There were statistically significant differences between the lipodystrophy group and the control group in BMD at the total hip (P= 0.002) and the total body (P= 0.005) but not the lumbar spine (P= 0.136). Adjusting for the differences in age, body weight, fat mass or lean mass between the groups did not change these results.

**Table 2.4: Baseline characteristics of HIV-infected group in the presence or absence of lipodystrophy**

	Absent (N=26)	Present (N=33)	P
Age	47.7 (8.1)	52.1 (8.1)	0.043
Height (cm)	176.4 (6.3)	177.0 (6.0)	0.73
Weight (kg)	76.7 (9.7)	77.5 (10.8)	0.77
BMI (kg/m <sup>2</sup> )	24.7 (3.3)	24.7 (2.9)	0.99
Fat Mass (kg)	15.4 (8.7)	13.9 (6.5)	0.45
Lean Mass (kg)	56.1 (6.3)	59.0 (6.8)	0.11
Percent Fat	20.6 (9.6)	18.4 (6.3)	0.29

Data are mean (SD), P for difference between groups.

## **Discussion:**

In this cross-sectional study, there was no consistent evidence that BMD in HIV-infected Caucasian men treated with HAART was lower than that of healthy age-matched controls, after allowance for weight differences. The unadjusted spinal and total body BMD were similar in the two groups, although there was a small (5.5%) decrement in BMD at the total hip in the HIV-infected group. When we restricted the analysis to the prevalence of low BMD, we found no differences between the groups in the rates of osteopenia or osteoporosis, and HIV infection did not predict the presence of low BMD. There were significant differences between the HIV-infected group and the control group in two important osteoporosis risk factors, namely body weight and smoking exposure. HIV-infected men were on average 6 kg lighter, and reported greater overall smoking exposure, than the controls. Body weight is a major determinant of BMD in men [10], while smoking has been associated with dose-dependent reductions in BMD at all sites [137]. When we adjusted for the differences in body weight and smoking exposure between the groups, BMD was similar at all three sites in the study groups. There were no clinically important differences in calcitropic hormones and bone-related laboratory parameters between the HIV-infected group and the control group. Although there were small, statistically significant differences between the groups in 25OHD, ALP and albumin-adjusted serum calcium, the mean values for both groups were within the reference range and the differences are unlikely to be clinically or biologically significant. These biochemical results are consistent with our finding that BMD is not significantly compromised in HIV-infected men treated with HAART.

Our BMD results differ from those of several published studies in adults. In those studies, HIV infection has consistently been associated with lowered BMD, and/or higher than expected prevalence of osteopenia or osteoporosis [5, 24-37, 39-56]. A possible explanation for the difference in results between this study and previous studies is differences in the characteristics of the HIV-infected groups, though there is little evidence to support this. The HIV-infected cohort in this study had similar baseline HIV-related clinical characteristics (CD4 count and viral load prior to, and during, treatment with HAART, duration of infection, and prevalence of AIDS-defining illness) to the HIV-infected groups in the other studies in which those data were reported, and to a nationwide New Zealand cohort of HIV-infected men [138]. There may be differences in the nature and type of antiretroviral exposure between our HIV-infected cohort and those in other studies. The low number of changes of medication regimen per HIV-infected subject in our cohort may reflect relatively conservative prescribing. Data from *in-vitro* and animal studies suggest that different antiretroviral agents may exert divergent effects on skeletal tissue [123, 124]. Definitive demonstration of the putative skeletal effects of particular antiretroviral agents in studies of BMD in humans conducted to date has been impossible because of the large number of drug regimens employed in the cohorts studied.

Another possible explanation for the discrepancy of results between this and other studies is that the HIV-infected participants in this study have had a longer duration of treatment with HAART, around 20 months longer than in other studies that have reported similar data. Thus, participants in this study may have had better nutritional status for a longer period of time, and have regained more of the body weight that was initially lost with advancing HIV infection, than HIV-infected participants in earlier studies. On average the HIV-infected men in our cohort lost 3.8 kg of body weight between their first clinic visit and their lowest recorded weight. However, by the time of entry to this study they had regained this lost weight, and were on average 1 kg heavier than their first recorded clinic body weight. Weight gain has consistently been shown to be associated with an increase in BMD in various populations [10, 139, 140], and body weight in our cohort increased by an average of 4.6 kg during 4.3 years of treatment with HAART. The hypothesis that a longer period of improved nutrition is responsible for the finding that BMD is not compromised in our HIV-infected cohort is consistent with data from 11 longitudinal studies of HIV-infected cohorts treated with HAART that reported either stable or increasing BMD over time [29, 34, 37, 40, 43, 68, 70-74]. The persisting weight difference we observed between the groups at the time of the

current analysis may be attributable to the HIV-infected group having not fully regained the body weight lost because of HIV infection. Alternatively, it may be partly attributable to the higher prevalence of smoking in the HIV-infected group, since smoking exposure is inversely related to body weight [141]. In addition, the majority of the HIV-infected group identified as homosexual, and homosexual men have been reported to have lower body weight [142], be more concerned with body shape and appearance [143], and have higher rates of smoking [144] than heterosexual men.

A further possible explanation for the discrepancy of results between this and other studies is differences in methodology related to the generation and analysis of control group data. 17 of the previous studies did not include a locally-generated control group, and instead relied on the densitometer reference database for comparative purposes [25, 29, 31, 32, 34, 35, 37, 44-46, 48, 50-54, 56]. In the 16 studies that did include a control group, two did not report body weight or age [33, 43], one did not describe the number or gender of the controls [24], seven included fewer than 50 controls [5, 27, 28, 30, 33, 36, 42], three had a control group with a mean BMI of 23 or less [5, 26, 30], four did not adjust for significant weight differences between groups [24, 36, 47, 49], and two studies appeared to be duplicate publications of similar study groups [39, 40]. Thus, only three studies had an adequately described control group of moderate size [39, 41, 55]. In those studies, the HIV-infected group had a 1-2 kg/m<sup>2</sup> lower BMI [39, 41] or a 9 kg lower body weight [55] than the controls, but the BMD difference between groups persisted despite adjusting for this BMI or weight difference. Our sizeable control group was drawn from the same community as the HIV-infected subjects, and we were able to adjust adequately for confounders known to influence BMD.

Approximately half the HIV-infected group had evidence of lipodystrophy on clinical examination. This sub-group had lower BMD at each site than those without evidence of lipodystrophy but the differences were only statistically significant at the total body. Similarly, the group with lipodystrophy had lower BMD in comparison to the control group, but the differences only reached statistical significance at the total hip and total body. These differences persisted despite adjustment for the differences in age, and body composition between the groups. These results are similar to those from three other studies which reported reduction in BMD in HIV patients with lipodystrophy [25, 28, 42]. The mechanism of the reduction in BMD is not clear, although our results suggest it is not simply due to differences in body composition.

There are limitations to our study. Both groups of participants were volunteers. Participants may have chosen to take part because they were more aware of health issues and healthier than average, and therefore have higher BMD. Conversely, participants may have chosen to take part because they believed they were at higher risk of osteoporosis because of family history or background of osteoporosis, and therefore had lower BMD. The study is moderately sized, with adequate (>80%) power to detect a difference of 0.4 SD in BMD between groups. Thus, there is the possibility that there is a small difference in BMD between groups that was not detected (Type II error), but if so, the magnitude of the difference is unlikely to be biologically significant. There was a densitometer change during the study which may have introduced bias to the results, however the accuracy of the predicted results compared to actual results during the validation stage of the interconversion procedure suggests that any such bias is likely to be minimal.

In conclusion, BMD is not significantly reduced in HIV-infected Caucasian men treated with HAART for about four years. We speculate that differences in body weight, including loss that occurs in the early stages of HIV infection and increases in response to treatment with HAART, may have confounded the analysis of previous cross-sectional studies of BMD in HIV disease. Our data suggest that osteoporosis is not a significant health issue for HIV-infected Caucasian men treated with long term HAART, and that routine monitoring of BMD is not necessary.

# **Chapter 3: Annual zoledronate increases bone mineral density in HAART-treated HIV-infected men: a randomised controlled trial**

## **Introduction:**

As discussed in Chapter 1, many cross-sectional studies have reported low BMD or higher than expected rates of osteopenia and osteoporosis in people infected with HIV [5, 24-37, 39-56], although not all studies have found such associations, as reported in Chapter 2. The cause of the association between HIV infection and low BMD is not known but appears to be multifactorial, as discussed in Chapter 1. This association has been attributed to HIV infection itself, or to treatment with protease inhibitors or HAART [91, 93]. Patients infected with HIV may also potentially be at risk of osteoporosis because of increased exposure to more traditional risk factors for osteoporosis such as chronic illness, low body weight, and hypogonadism [83].

Bisphosphonates are widely used and highly effective agents for the treatment and prevention of osteoporosis [145]. In patients infected with HIV, three small, randomised, open-label studies of weekly oral 70 mg alendronate treatment for 48-96 weeks have produced conflicting results. One study reported increases in BMD at the spine and hip but only significant between-groups differences favouring alendronate at the trochanter [136], one study reported between-groups differences in BMD favouring alendronate at the spine but not other sites [135], and one study reported no between-groups differences in BMD at either the spine or the hip [134].

Zoledronate is a potent third-generation bisphosphonate. Recently Reid et al. reported that intravenous administration of a single dose of 4 mg of zoledronate produced significant increases in BMD and suppression of bone turnover markers over the following 12 months in post-menopausal women with low BMD [146]. The effects of annual administration of zoledronate in men, in patients infected with HIV, or in any population beyond 12 months of follow-up have not yet been reported. Therefore we performed a two-year randomised, double-blind, placebo-controlled trial of annual administration of zoledronate to determine the effects on BMD and bone turnover markers in men infected with HIV who were taking HAART.

## **Methods:**

### ***Participants:***

Between February 2003 and March 2004, all HIV-infected men who attended infectious disease clinics at our institution were approached by their primary physician to participate. Men were eligible to participate if they had been treated with HAART for at least three months and had a BMD T score of less than -0.5 at the lumbar spine or total hip. HAART was defined as an HIV treatment regimen containing at least three antiretroviral agents. We excluded men with significant renal, hepatic or thyroid dysfunction, concurrent major systemic illness including malignancy, metabolic bone disease, or current use of a bisphosphonate or systemic glucocorticoids. Approximately 220 men were approached and 72 agreed to have a screening measurement of bone density. 45 men had a BMD T score less than -0.5 at the lumbar spine or total hip. One man was excluded because of liver cirrhosis, and one because of newly diagnosed primary hypogonadism. Thus 43 men were enrolled in the two-year study.

Figure 3.1 shows the flow of participants through the trial. One man withdrew from the study for personal reasons after randomisation but prior to administration of study medication, one died of unknown causes, and four emigrated during the study. Thus 37 men completed two years of follow-up. Two men (both received zoledronate) stopped intravenous study medication because of influenza-like illnesses after the first administration of study drug, but remained in the study. However, following the intention to treat principle, all 43 men enrolled in the trial were included in the analyses. The study received ethical approval from the Auckland Ethics Committee and was registered with the Australian Clinical Trials Registry, ACTRN012605000208606. All participants gave written, informed consent.

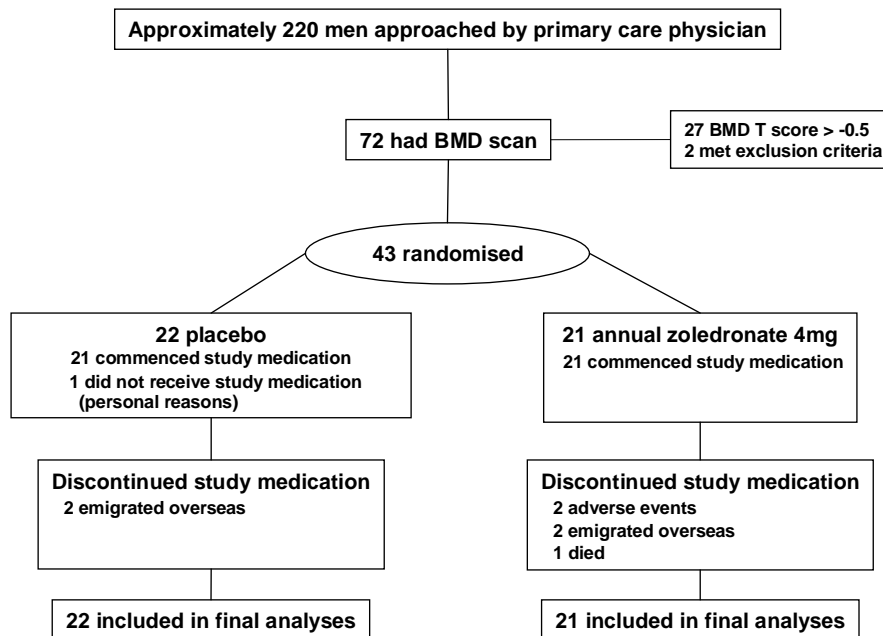
### ***Protocol:***

Participants were randomly allocated to receive an annual administration of either zoledronate 4 mg, given as a 15 minute intravenous infusion in 100mL of 0.9% NaCl, or placebo for two years. Randomisation was performed in blocks of variable size, using computer-generated random numbers (MS-Excel 2000). Subject numbers were allocated and medication dispensed by staff who had no direct contact with the participants. All study staff and participants remained blinded to treatment allocation throughout. In addition, all participants



received a supplement of 400 mg/day of elemental calcium and 1.25 mg/month of vitamin D (cholecalciferol). Participants were seen every six months during the study period.

**Figure 3.1: Flow of subjects through the study**



**Measurements:**

BMD and body composition were measured every six months at the lumbar spine, proximal femur and total body using a Lunar Expert DXA (GE Lunar, Madison, WI). At baseline and two years, vertebral morphometry was also performed. Daily calcium intake was assessed at baseline using a validated questionnaire [147]. At baseline, 3 months, 12 months, and 24 months fasting blood and second-voided morning urine samples were collected. The following assays were used: serum 25OHD was measured by RIA (DiaSorin, Stillwater, MN); serum PTH and serum testosterone by electrochemiluminescence immunoassays (E170, Roche); urine NTx by ELISA (Ostex International Inc., Seattle, WA). At baseline, measurements of biochemistry, calcium metabolism, testosterone, HIV parameters (CD4 count, viral load) and bone turnover were performed. Measurements of bone turnover were repeated at 3, 12 and 24 months, and HIV parameters at 24 months.

## **Statistics:**

The primary endpoint was the difference between groups in the change in BMD at the spine over two years. This study had power of at least 80% to detect a between-groups difference in percent change from baseline BMD of the lumbar spine of 4% ( $\alpha= 0.05$ ). Differences between groups for continuous variables were assessed using Student's t-test and for categorical groups using the Chi-Square test. Pearson correlation analysis was used to test for significant linear correlations between variables. BMD data were analysed as absolute changes from baseline values, although results are presented as percentage change from baseline for ease of interpretation. A mixed models approach to repeated measures (ANCOVA) was used to examine the time course of response in treatment and control arms for BMD measurements, bone turnover markers, and measurements of body composition. Changes in variables between time-points were further explored using the method of Tukey. Inspection of plots for urine NTx showed that data were not normally distributed. Therefore these data were ranked and the ranked values were analysed using a non-parametric mixed models approach, although data are presented using medians and confidence intervals (binomial method: Confidence Interval Analysis, version 2.1.1) for ease of interpretation. All tests were two-tailed and statistical significance was set at  $P < 0.05$ . All statistical analyses were carried out using the SAS software package (SAS Institute, Cary, NC version 9.1)

## **Results:**

Table 3.1 shows the baseline characteristics of the study participants, and Table 3.2 shows the HIV-related clinical characteristics. There were no statistically significant differences between the groups in these parameters. There were no significant HIV-related clinical events for any of the participants during the study. 34/43 participants were treated with HAART regimens which achieved consistent suppression of HIV replication (viral load less than 1.7 log copies/mL). Nine participants were treated with regimens which were only partially or intermittently effective due either to infection with HIV resistant to antiretroviral medications or to inadequate treatment adherence. 32/34 participants with sustained suppression of HIV replication had CD4 counts greater than 200 cells/ $\mu$ L, while 2/9 subjects with intermittently effective treatment had at least one CD4 count less than 200 cells/ $\mu$ L.

**Table 3.1: Baseline anthropometric, BMD, biochemical parameters and other characteristics of the groups**

<b>Characteristic</b>	<b>Placebo N=22</b>	<b>Zoledronate N=21</b>
Age (years)	48.8 (9.0)	49.5 (9.0)
European Descent	82%	81%
Height (cm)	176 (8)	176 (4)
Weight (kg)	75 (12)	73 (10)
BMI (kg/m <sup>2</sup> )	24.6 (3.4)	23.5 (3.1)
Percent Fat	20.8 (5.6)	17.2 (6.7)
Fat Mass (kg)	15 (5)	12 (7)
Lean Mass (kg)	55 (9)	56 (7)
Previous Smoker	68%	52%
Current Smoker	27%	24%
Dietary Calcium (mg/day)	854 (600)	963 (699)
GGT (U/L)	59 (54)	78 (57)
Serum Adjusted Calcium (mmol/L)	2.22 (0.08)	2.27 (0.09)
PTH (pmol/L)	5.2 (2.3)	4.0 (1.7)
25OHD (nmol/L)	58 (28)	70 (25)
Testosterone (nmol/L)	17 (6)	20 (11)
L1-L4 BMD (g/cm <sup>2</sup> )	1.11 (0.16)	1.15 (0.11)
T score L1-L4	-0.9 (1.3)	-0.6 (0.9)
Total femur BMD (g/cm <sup>2</sup> )	0.94 (0.11)	0.94 (0.07)
T score Total Femur	-1.2 (0.9)	-1.2 (0.6)
Total Body BMD (g/cm <sup>2</sup> )	1.13 (0.09)	1.12 (0.07)

Data are mean (SD) or percentage. There were no statistically significant differences between the groups.

**Table 3.2: HIV-related clinical characteristics of the groups**

<b>Characteristic</b>	<b>Placebo N=22</b>	<b>Zoledronate N=21</b>
Time since diagnosis (years)	7.8 (5.5)	8.3 (5.6)
AIDS defining illness	27%	38%
Lipodystrophy <sup>a</sup>	50%	71%
First recorded body weight (kg) <sup>b</sup>	73 (13)	73 (13)
Body weight nadir (kg) <sup>b</sup>	70.6 (13.7)	69.5 (13.3)
CD4 count (cells/μL)		
nadir <sup>b,c</sup>	130 (82)	131 (97)
study entry <sup>c</sup>	521 (250)	559 (235)
after 2 years <sup>c</sup>	520 (252)	509 (208)
Viral load (log copies/mL)		
peak <sup>b</sup>	4.9 (0.5)	4.4 (0.9)
study entry	1.8 (0.5)	2.1 (0.9)
after 2 years	1.9 (0.8)	2.3 (1.1)
Duration of treatment (months)	55 (44)	67 (40)
Duration of HAART (months) <sup>d</sup>	44 (24)	52 (22)

Data are mean (standard deviation) or percentage. There were no statistically significant differences between the groups.

<sup>a</sup> Lipodystrophy was defined as evidence of peripheral fat loss or central fat accumulation on clinical examination.

<sup>b</sup> Nadir/peak refers to lowest/highest value while under regular follow up prior to study entry.

<sup>c</sup> Reference range: 500-1650 cells/μL

<sup>d</sup> HAART was defined as an HIV treatment regimen containing at least three antiretroviral agents.

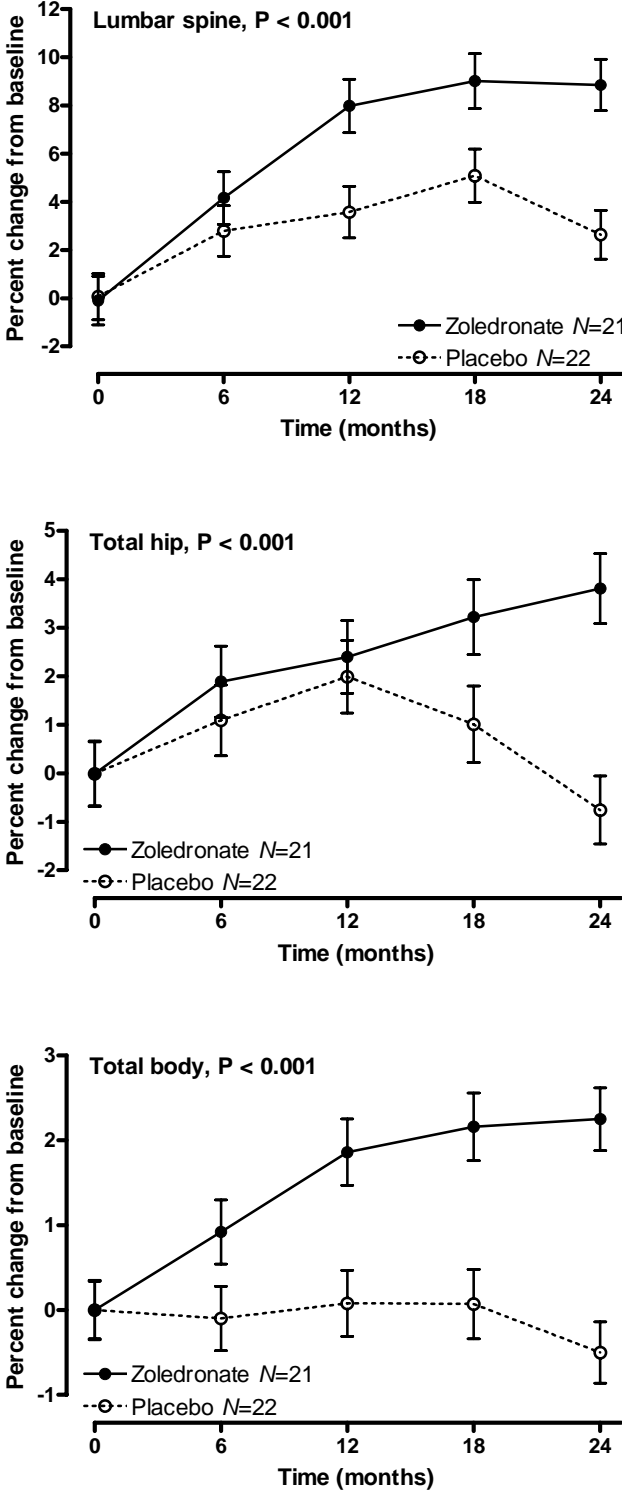
The effect of zoledronate on BMD is shown in Figure 3.2. At the lumbar spine, BMD increased by 8.9% over two years in the zoledronate group compared to an increase of 2.6% in the control group ( $P < 0.001$ ). At the total hip, BMD increased by 3.8% over two years in the zoledronate group compared to a decrease of 0.8% in the control group ( $P < 0.001$ ). At the total body, BMD increased by 2.3% over two years compared to a decrease of 0.5% in the control group ( $P < 0.001$ ).

The effect of zoledronate on bone turnover markers is shown in Figure 3.3. In the zoledronate group, urine NTx levels decreased by 61% from baseline at three months ( $P < 0.001$ ) and thereafter levels remained stable within the lowest tertile of the normal range for men and pre-menopausal women. Urine NTx levels in the placebo group were stable over the two years ( $P > 0.9$ ). The between-groups differences in urine NTx over two years were statistically significant ( $P < 0.001$ ). In the zoledronate group, total serum ALP decreased by 21% from baseline at three months ( $P < 0.001$ ) and thereafter levels remained stable. ALP levels were stable in the placebo group over the two years. Levels of urine NTx ( $P = 0.66$ ) and ALP ( $P = 0.97$ ) measured at 24 months did not decline from their respective levels measured at 12 months despite a second dose of zoledronate, administered at 12 months.

Both groups tended to gain weight [mean change from baseline (95% CI) 0.5 kg (-0.8 to 1.7),  $P = 0.45$  for zoledronate group, and 1.3 kg (0.0 to 2.5),  $P = 0.05$  for placebo group] and fat mass [mean change from baseline 0.5 kg (-0.8 to 1.8),  $P = 0.46$  for zoledronate group, and 1.7 kg (0.4 to 3.0),  $P = 0.01$  for the placebo group] during the study, whereas lean mass remained unchanged [mean change from baseline 0.0 kg (-0.9 to 0.9),  $P = 0.99$  for zoledronate group, and 0.1 kg (-0.8 to 0.9),  $P = 0.84$  for placebo group]. There were no significant between-groups differences in the change from baseline for either weight or body composition ( $P > 0.2$ ), and change in body weight or body composition from baseline did not correlate with change in BMD at any site in either group ( $P > 0.2$ ).

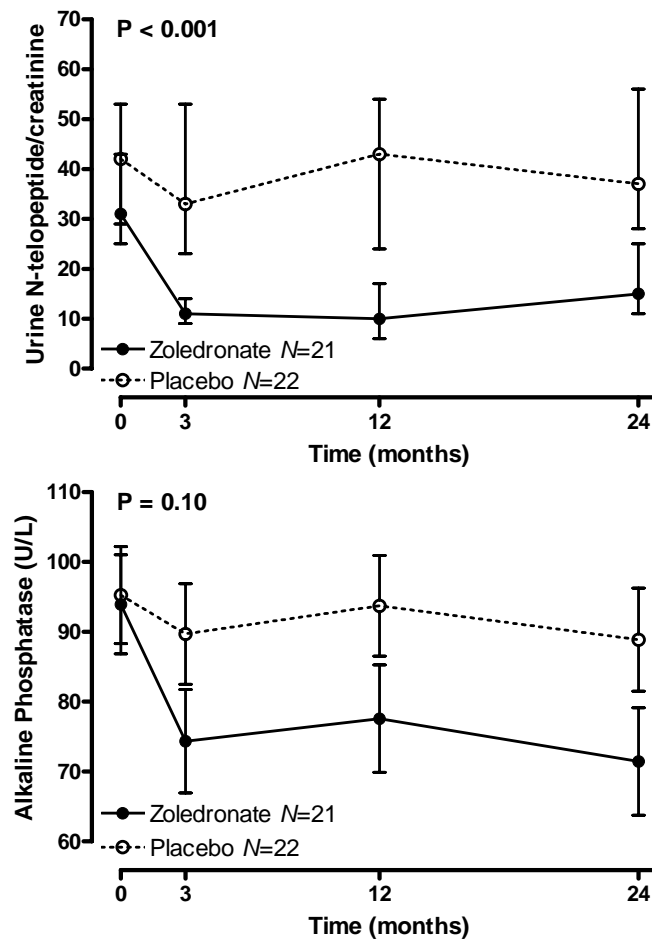
Zoledronate was generally well tolerated. Two men in the zoledronate group experienced acute-phase reactions that led them to discontinue study treatment after the first infusion, but no other treatment-related adverse effects were reported. One man who received placebo suffered a vertebral fracture at month 21 of the study. There were no other clinical fractures reported, and no other participants had fractures detected by vertebral morphometry at baseline or at two years.

**Figure 3.2: The effects of 4 mg annual zoledronate or placebo on BMD at the lumbar spine, total hip, and total body in HIV-infected men**



Bone densities are expressed as mean (SE) percent of initial values. P values are for the time-treatment interaction.

**Figure 3.3: The effects of 4 mg annual zoledronate or placebo on bone turnover markers in HIV-infected men**



Data are median (98% CI) for urine NTx and mean (SE) for ALP. P values are for the time-treatment interaction. The units of urine NTx are nM bone collagen equivalents / mmol urine creatinine.

## **Discussion:**

The current study provides evidence for efficacy of zoledronate in the treatment of HIV-infected men who manifest significant bone loss. We found that zoledronate significantly increased BMD at the lumbar spine, hip, and total body in HIV-infected men treated with HAART, and that the between-groups differences in BMD at all sites tended to increase throughout the study. Bone resorption decreased substantially by three months and remained stable thereafter. This is the first report of the effects on BMD of annual administration of the potent bisphosphonate, zoledronate, in men, in HIV-infected subjects, or with drug administration beyond 12 months. These results are consistent with those of Reid et al. who reported an increase in BMD at the lumbar spine of 5% and at the femoral neck of 2.5%, and

a reduction in bone resorption markers of 50-60% in post-menopausal women at 12 months following a single administration of 4 mg of intravenous zoledronate [146]. Thus, our findings extend the beneficial actions of zoledronate on BMD to men, and to HIV-infected subjects, and suggest that there are ongoing benefits from annual administration of zoledronate for at least 24 months. The annual administration of this agent is an additional benefit in HIV-infected patients, since many HAART regimens involve a complicated daily schedule of ingestion of several different medications, which is likely to impact adversely on compliance with other treatments.

There are few published studies on the use of bisphosphonates in men. Two studies have reported on the effect of daily 10 mg alendronate on men with primary osteoporosis [148, 149]. Both studies reported significant increases in BMD from baseline over two years of treatment with increases of 7.1-10.1% at the lumbar spine and 3.5-5.2% at the femoral neck. These increases in BMD were associated with a 7% reduction in vertebral fractures compared to placebo [148], and a 11% reduction compared to alfacalcidol [149]. We observed similar changes in BMD to these two studies suggesting that annual administration of zoledronate may have similar efficacy to daily treatment with alendronate.

An important finding of the current study is that suppression of bone resorption remains stable and does not progressively decline during the second year of zoledronate therapy. Recently, concerns have been raised about potential over-suppression of bone turnover during long term bisphosphonate therapy. Such over-suppression could increase susceptibility to fractures that fail to heal or heal poorly [150, 151]. We found that annual administration of zoledronate led to a rapid 60% decrease in urine NTx levels after the first dose, but thereafter median levels of urine NTx remained stable, within the lower part of the normal range, between 3 and 24 months. Epidemiological evidence suggests that suppression of bone resorption into this range is associated with optimal anti-fracture efficacy conferred by anti-resorptive therapy [152].

There are three previous studies of bisphosphonate treatment in patients infected with HIV. All were randomised, open-label trials of alendronate 70 mg weekly, and produced conflicting results. Guaraldi et al. reported a 52 week trial of alendronate compared to placebo in 41 HIV-infected patients who had been taking HAART for at least six months and had a BMD T score less than -1 [134]. All participants took calcium carbonate 1000 mg daily and vitamin D 500 IU daily. There were no statistically significant between-groups differences in bone turnover markers or BMD at the spine or femoral neck. Mondy et al. reported a 48 week trial

of alendronate compared to placebo in 31 HIV-infected patients treated with HAART for at least six months who had a lumbar spine BMD T score less than -1 [135]. All participants received 1000 mg calcium and 400 IU vitamin D daily. BMD at the spine increased by 5.2% in the alendronate group compared with 1.3% in the control group ( $P < 0.05$ ), whereas there were no between-groups differences at the femoral neck, trochanter, total hip or total body. Bone turnover markers decreased in both groups but the between-groups difference was only statistically significant for bsALP. Negrodo et al. reported a 96 week trial of alendronate with dietary counselling to ensure a daily calcium intake of at least 1200 mg compared to dietary counselling alone in 25 HIV-infected patients taking HAART with BMD T score less than -2.5 [136]. There were increases in BMD at the spine and hip in the alendronate group whereas BMD tended to decrease in the control group. The between-groups differences in BMD were only statistically significant at the trochanter. The conflicting results between these studies may be due to the small size of the studies, or the differences in study design and duration of follow up. Our study provides rigorous evidence that bisphosphonate therapy improves BMD in HIV-infected subjects.

In summary, the annual administration of 4 mg zoledronate is a potent and effective treatment for bone loss in men infected with HIV. It produces substantial increases in BMD and suppression of bone turnover that persist for at least two years. The only previous report of annual zoledronate treatment demonstrated benefits for post-menopausal women with low BMD over 12 months of follow-up. Our findings extend the potential benefits of annual treatment with zoledronate to include men, and low BMD in association with HIV infection, and show that benefits persist for at least two years. Annual administration of zoledronate is a convenient and effective option for the treatment or prevention of bone loss for HIV-infected men with low BMD.



## **Chapter 4: Bone mineral density remains stable in HAART-treated HIV-infected men over two years**

### **Introduction:**

As discussed in Chapters 1 and 2, there is an unexplained disparity between the findings of the majority of the cross-sectional studies, which have reported low BMD or higher than expected rates of osteopenia and osteoporosis in people infected with HIV [5, 24-37, 39-56], and those of the longitudinal studies, which, in contrast, have reported stable or increasing BMD over time in cohorts treated with HAART [29, 34, 37, 40, 43, 70-74]. However, many of these longitudinal studies have had methodological shortcomings which complicate their interpretation, in particular the lack of a control group or a short duration of follow-up. As discussed in Chapter 1, the association between HIV infection and low BMD in cross-sectional studies has been attributed to HIV infection itself, treatment with protease inhibitors or HAART [91, 93], or to increased exposure to more traditional risk factors for osteoporosis such as chronic illness, low body weight, and hypogonadism [83]. However, the absence of accelerated bone loss over time in the longitudinal studies suggests that the impact of these factors is not sustained.

In Chapter 2, I reported that Caucasian HIV-infected men were 6.3 kg lighter than age-matched healthy controls, and that there were no differences in BMD between the groups after adjusting for this weight difference. We hypothesised that differences in body weight, including losses that occur in the early stages of HIV infection and increases in response to treatment with HAART, may have confounded the analysis of previous cross-sectional studies of BMD in HIV disease. This hypothesis predicts that BMD changes over time would be similar in HIV-infected groups and control groups. Here we report on the longitudinal changes in BMD over two years in a cohort of HAART-treated HIV-infected men and a comparable control group of healthy men.

### **Methods:**

#### ***Subjects:***

All HIV-infected men (approximately 220) who attended infectious disease clinics at our institution were approached by their primary physician to participate in a clinical study of

bisphosphonate therapy, and 72 men agreed to attend a screening visit. Men were eligible to participate if they had been treated with HAART for at least three months and were excluded if they had significant renal, hepatic or thyroid dysfunction, concurrent major systemic illness including malignancy, metabolic bone disease, or current use of a bisphosphonate or systemic glucocorticoids. All participants then had a measurement of BMD. 43 men with BMD T score less than -0.5 were enrolled in the intervention study described in Chapter 3. The other 29 men (with T score greater than -0.5) were invited to have a repeat measurement of BMD after two years, and are the focus of this report. In addition, we recruited a group of 32 healthy men by workplace advertisements to act as controls. The same exclusion criteria that were applied to the HIV-infected men were also applied to the control group except that there were no BMD criteria for the controls. All 29 HIV-infected men and 32 controls had a measurement of BMD at baseline. Here we report on the 23 HIV-infected men and 26 controls who had a follow-up BMD measurement after two years. None of the participants took any bone active medication during the two-year follow-up period. The study received ethical approval from the Auckland Ethics Committee, and all participants provided written, informed consent.

### ***Measurements:***

Height was measured at baseline using a Harpenden stadiometer and weight was recorded using electronic scales. All men supplied a fasting blood sample and a fasting second-voided morning urine sample at the baseline visit. The following assays were used: serum 25OHD was measured by RIA (DiaSorin, Stillwater, MN); serum PTH and serum testosterone by electrochemiluminescence immunoassays (E170, Roche); urine NTx by ELISA (Ostex International Inc., Seattle, WA). Body composition and BMD of the lumbar spine, proximal femur and total body were measured using a Lunar Expert dual-energy x-ray absorptiometer (GE Lunar, Madison, WI).

### ***Statistics:***

Comparisons were made between groups according to HIV status for continuous variables using Student's t-test, and for categorical variables using the Chi-square test. Paired t-tests were used to compare baseline and final variables. Pearson correlation analysis was used to test for significant linear correlations between variables. Significance level was set at  $P < 0.05$  and all tests were two-tailed. BMD data were analysed as absolute changes from baseline values, though results are presented as percentage change from baseline for ease of

interpretation. A mixed models approach to repeated measures was used to examine the time course of response in the HIV-infected men and the controls for measurements of BMD. Changes in BMD between time points were further explored using the method of Tukey. All statistical analyses were obtained using the SAS software package (SAS Institute, Cary, NC version 9.1).

## **Results:**

Descriptive and biochemical characteristics of the study groups are summarised in Table 4.1. There were no statistically significant differences in these characteristics between the groups at baseline except for increased past and current smoking rates in the HIV-infected men. HIV-related clinical characteristics are shown in Table 4.2. There were no significant HIV-related clinical events for any of the participants during the study. 17/23 subjects were treated with HAART regimens which achieved consistent suppression of HIV replication (viral load less than 1.7 log copies/mL), while 6/23 subjects were treated with regimens which were only partially or intermittently effective due either to infection with HIV resistant to antiretroviral

**Table 4.1: Characteristics of the study groups at baseline and two years**

Characteristic	HIV N=23			Controls N=26		
	Baseline	Follow up	P	Baseline	Follow up	P
Age (years)	47.0 (9.4)			44.9 (10.9)		
European descent (%)	82			100		
Weight (kg)	76.0 (10.2)	75.9 (11.2)	0.99	80.2 (10.6)	82.2 (11.5)	0.003
BMI (kg/m <sup>2</sup> )	24.4 (3.3)	24.5 (3.4)	0.70	25.1 (3.0)	25.8 (3.3)	0.001
Fat mass (kg)	14.1 (8.5)	12.3 (7.6)	0.05	15.7 (7.0)	17.8 (8.3)	0.007
Lean mass (kg)	56.9 (6.6)	58.2 (7.3)	0.13	58.8 (5.9)	58.7 (6.2)	0.77
Percent fat	19.0 (8.9)	16.5 (8.2)	0.01	20.4 (6.8)	22.4 (7.9)	0.01
Smoke previously (%)	65			27		
Smoke currently (%)	43			4		
Serum adjusted calcium (mmol/L)	2.26 (0.12)			2.26 (0.08)		
PTH (pmol/L)	3.8 (1.5)			4.1 (2.1)		
25OHD (nmol/L)	73.0 (26.4)			81.6 (20.1)		
Testosterone (nmol/L)	18.3 (6.5)			17.2 (4.7)		
Urine NTx (nmol BCE/mmol Cr)	35.0 (16.8)			32.2 (11.4)		
L1-4 BMD (g/cm <sup>2</sup> )	1.36 (0.16)			1.27 (0.17)		
T score L1-4	1.1 (1.3)			0.4 (1.4)		
Total hip BMD (g/cm <sup>2</sup> )	1.15 (0.09)			1.11 (0.14)		
T score Total hip	0.4 (0.7)			0.1 (1.1)		
Total body BMD (g/cm <sup>2</sup> )	1.24 (0.07)			1.23 (0.08)		

Data are mean (SD) or percentage, P values are for within-group differences between baseline and final values. There were no significant differences in the baseline characteristics between groups except for smoking previously (P= 0.01) and smoking currently (P= 0.001). NTx was measured on a fasting second-void morning urine sample and its units are nmol of bone collagen equivalents (BCE) / mmol of creatinine (Cr).

medications or to inadequate treatment adherence. All subjects with sustained suppression of HIV replication had CD4 counts consistently greater than 200 cells/ $\mu$ L, while 2/6 subjects with intermittently effective treatment had at least one CD4 count less than 200 cells/ $\mu$ L.

**Table 4.2: Clinical characteristics of HIV-infected group**

<b>Time since diagnosis (years)</b>	8.2 (4.7)
<b>AIDS defining illness</b>	30%
<b>Lipodystrophy<sup>a</sup></b>	30%
<b>First recorded body weight (kg)<sup>b</sup></b>	76.1 (13.4)
<b>Body weight nadir (kg)<sup>b</sup></b>	70.5 (9.4)
<b>CD4 count (cells/<math>\mu</math>L)</b>	
<b>nadir<sup>b,c</sup></b>	138 (119)
<b>study entry<sup>c</sup></b>	490 (180)
<b>at 2 years<sup>c</sup></b>	470 (230)
<b>Viral load (log copies/mL)</b>	
<b>peak<sup>b</sup></b>	4.7 (0.6)
<b>study entry</b>	2.3 (1.1)
<b>2 years</b>	2.4 (1.3)
<b>Duration of treatment (months)</b>	72.5 (40.6)
<b>Duration of HAART (months)<sup>d</sup></b>	53.4 (24.2)

Data are mean (SD) or percentage.

<sup>a</sup> Lipodystrophy was defined as evidence of peripheral fat loss or central fat accumulation on clinical examination.

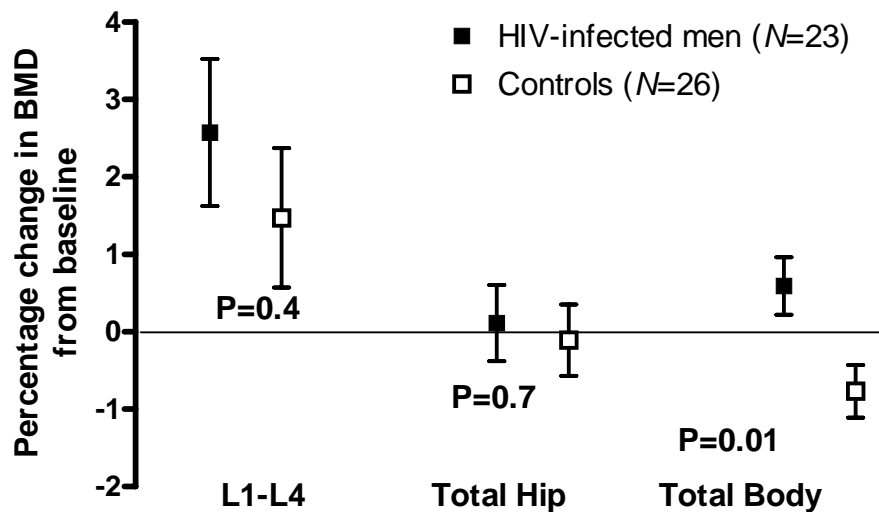
<sup>b</sup> All patients were followed regularly at infectious disease clinics after diagnosis of HIV infection was made. Values refer to data extracted from infectious disease clinic notes. Body weight was first recorded 66 (39) months [mean (SD)] prior to study entry and body weight nadir was 43 (35) months prior to study entry.

<sup>c</sup> Reference range: 500-1650 cells/ $\mu$ L

<sup>d</sup> HAART was defined as an HIV treatment regimen containing at least three antiretroviral agents.

Figure 4.1 shows the percentage change in BMD at the lumbar spine, total hip, and total body after two years in the HIV-infected men and controls. BMD increased from baseline in the HIV-infected men by 2.6% at the lumbar spine ( $P= 0.05$ ), 0.1% at the total hip ( $P> 0.99$ ), and 0.6% at the total body ( $P= 0.39$ ), while in the control group BMD increased by 1.5% at the lumbar spine ( $P= 0.37$ ), and decreased by 0.1% at the total hip ( $P> 0.99$ ), and 0.8% at the total body ( $P= 0.13$ ). The between groups-difference was statistically significant at the total body ( $P= 0.01$ ), where the change in BMD was more positive in the HIV-infected group. When we adjusted for the differences between groups at baseline in body weight and smoking exposure, the results were similar. (After adjusting for smoking status or body weight, there were increases in BMD of 2.6% at the lumbar spine, 0.1% at the total hip, and 0.6% at the total body in the HIV-infected group, and increases of 1.5% at the lumbar spine and decreases of -0.1% at the total hip and -0.8% at the total body).

**Figure 4.1: The change in BMD from baseline at the lumbar spine, total hip, and total body in HIV-infected men and controls**



Bone densities are expressed as mean (SE) percent of initial values.

Table 4.1 shows the changes in body weight and body composition during the study. In the HIV-infected men, body weight and BMI remained similar while fat mass and percent fat decreased and there was a trend toward an increase in lean mass. In the controls body weight, BMI, fat mass and percent fat all increased while lean mass remained stable. The between-groups differences for the change from baseline were only statistically significant for fat mass and percent fat ( $P < 0.001$ ).

Finally, we explored the associations between the change in BMD over two years and measures of body weight or body composition, urine NTx levels, or HIV-related parameters (CD4 count, viral load, duration of HAART) at baseline, or the changes in the anthropometric, body composition and HIV-related variables over two years. There were no consistent significant correlations between the changes in BMD at any site and any of these variables in either the HIV-infected men or the controls.

## **Discussion:**

In this prospective, two-year study, there was no evidence that BMD loss in HIV-infected men treated with HAART with BMD T score greater than -0.5 was greater than that of healthy controls. In fact, mean increases in BMD in the HIV-infected men were greater than those in the controls, with between-groups differences after two years of 0.2% to 1.4%, and the difference was significant at the total body, where measurement precision is greatest. This

BMD stability occurred despite there being differences between the HIV-infected men and the controls in two important osteoporosis risk factors, namely body weight and smoking exposure. The HIV-infected group were on average 4.2 kg lighter than the controls at baseline, and 6.3 kg lighter at two years, and had greater smoking exposure, as we previously had observed in Chapter 2. Body weight is a major determinant of BMD in men [10], while smoking has been associated with dose-dependent reductions in BMD at all sites [137]. Thus, BMD remained stable over time in the HIV-infected group compared to the controls despite higher prevalence of risk factors for accelerated bone loss.

Our findings are generally consistent with previous reports of longitudinal studies. There have been 11 previous longitudinal studies of BMD in HIV-infected, HAART-treated patients [29, 34, 37, 40, 43, 69-74]. However, only one study included a control group [40] and only seven studies had a duration of follow up of at least one year [34, 40, 43, 69, 70, 72, 74]. In these longitudinal studies, BMD was stable or increased over time, and treatment with protease inhibitors was not associated with accelerated bone loss [29, 34, 40], consistent with recent cross-sectional studies [25-28, 30, 32-36, 39, 41, 42, 44, 49, 55, 56]. Only two studies were large enough to assess factors associated with longitudinal changes. Mondy et al. reported that in 93 HIV-infected men and women (mean age 41 years, 87% male) the change in BMD at the spine correlated with the change in CD4 count while receiving HAART, and that participants with undetectable viral loads had the greatest increases in BMD [34]. Dolan et al. reported that in 100 HIV-infected women (mean age 41 years) CD4 count, measures of body weight, FSH level, urine NTx and baseline BMD were predictors of BMD changes over time [40]. However, this report is limited by a 75% dropout rate over the two-year study period. We did not find significant correlations between changes in BMD and measures of body weight, HIV-related parameters or bone turnover markers at baseline or in anthropometric or HIV-related variables over time, perhaps in part due to the smaller size of our study.

The data from our cross-sectional and longitudinal studies may resolve the discrepancy between the high prevalence of osteopenia in cross-sectional studies of HIV-infected people taking HAART [5, 24-37, 39-56] and the absence of accelerated bone loss over time in the longitudinal studies. One possible explanation is that weight loss occurs in association with advancing untreated HIV-infection, but following the initiation of HAART there is steady regain of this lost weight. Table 4.2 shows that in our cohort on average 6 kg of body weight was lost with advancing HIV infection, with nadir body weight occurring 10 months after

initiation of HAART, and subsequently, body weight increased by 6 kg during four years of treatment with HAART. Since body weight is a major determinant of BMD [10], and weight gain has consistently been shown to be associated with increases in BMD in various populations [10, 139, 140], BMD would be predicted to decrease with advancing untreated HIV-infection, and increase following the introduction of HAART. If this occurred, cross-sectional studies performed shortly after the introduction of HAART would report higher than expected rates of low BMD, whereas longitudinal studies would report stable or increasing BMD. In addition, cross-sectional studies performed after a longer time interval following the introduction of HAART would not report increased rates of low BMD. In our previous cross-sectional study described in Chapter 2, we found that there was a small reduction in BMD at the total hip in HIV-infected men compared to controls, but after adjustment for the 6.3 kg weight difference between the groups, there were no between-groups differences in BMD at any site. Our cohort of men had been treated with HAART for an average of 52 months, approximately 20 months longer than cohorts from other cross-sectional analyses, consistent with this hypothesis. It is also possible that some component(s) of HAART contributes to the improving BMD in HIV-infected cohorts, as several protease inhibitors have been demonstrated to influence bone cell function *in-vitro* [123] or *in-vivo* [29]. A further possibility is that the insulin resistance induced by HAART [88] may indirectly confer skeletal benefit [10].

Recently, three studies have reported the effects of treatment with alendronate on BMD in HIV-infected people [134-136], and the effects of treatment with zoledronate are described in Chapter 3. However, it remains uncertain what proportion of HIV-infected patients require treatment with bisphosphonates. Since there are inconsistent findings from cross-sectional and longitudinal studies of HIV-infected cohorts with regard to BMD, and there are very few reports of clinical fractures in association with HIV infection [127-129, 131], it is not certain that HIV-infected people are at increased risk of osteoporotic fractures. Therefore, in the absence of definitive evidence of increased skeletal morbidity in HIV-infected subjects, it seems reasonable to apply standard guidelines for treatment of patients with osteoporosis to HIV-infected subjects [153, 154].

There are limitations to our study. This was a small group of HIV-infected men with normal to above average BMD and so the results may not apply to HIV-infected men with low BMD. However, these results are consistent with data from an intervention study in which BMD

remained stable over a two-year period in the control group, all of whom had BMD T score less than -0.5, as described in Chapter 3. In our current study, both groups of participants were volunteers, and volunteer bias may have occurred. Participants may have chosen to take part because they were more aware of health issues and healthier than average, and therefore had higher BMD. Conversely, participants may have chosen to take part because they believed they were at higher risk of osteoporosis because of family history or background of osteoporosis, and therefore had lower BMD.

In conclusion, BMD remained stable or tended to increase over two years in a group of HIV-infected men treated with HAART. Taken together with our recent cross-sectional study reporting normal weight-adjusted BMD in HIV-infected men, our findings suggest that osteoporosis is not a significant health issue for HIV-infected men treated with HAART, and that routine monitoring of BMD is not necessary.



## Conclusions to Chapters 1-4:

In contrast to all other published cross-sectional analyses, we did not find evidence of reduced BMD or increased rates of osteopenia and osteoporosis in our cohort of HIV-infected Caucasian men compared to age-matched healthy controls, after adjusting for the differences in body weight between the groups. The HIV-infected cohort was on average 6.3 kg lighter than the control group, a finding consistent with other published literature. In addition, we found evidence of substantial decline in body weight with progression of untreated HIV infection that was reversed following the introduction of HAART. These changes in body weight are likely to have a significant impact upon BMD. Weight loss occurring with advancing HIV infection is likely to cause loss of BMD, but the loss of BMD is likely to be reversed following the weight gain that occurs after the introduction of HAART. Many previous studies did not take into account the lower body weight of HIV-infected patients or the temporal changes in body weight that occur prior to and following the introduction of effective antiretroviral therapy. We hypothesise that this failure to consider body weight may explain in part the difference between our results and those of previous studies.

We found that BMD remained stable over two years in our longitudinal analysis of BMD in HIV-infected men with baseline BMD T score greater than -0.5 in comparison to healthy controls. BMD also remained stable over two years in HIV-infected men with BMD T score less than -0.5 in the control arm of our intervention study with annual intravenous zoledronate. These findings of BMD stability and absence of accelerated bone loss over time are consistent with most, but not all, recently published longitudinal studies of BMD in HIV-infected men and women. The discrepancy between the high prevalence of osteopenia in cross-sectional studies of HIV-infected people taking HAART and the absence of accelerated bone loss over time in the longitudinal studies may be explained by the changes in body weight occurring in untreated and treated HIV infection. Weight loss in advancing untreated HIV infection is likely to cause loss of BMD. Therefore, cross-sectional studies performed shortly after the introduction of HAART would report higher than expected rates of low BMD. However, weight gain following the introduction of HAART is likely to cause regain of the lost BMD. Therefore, longitudinal studies would report stable or increasing BMD, and cross-sectional studies performed after a longer time interval following the introduction of HAART (such as our cross-sectional study) would not report increased rates of low BMD.

We found that annual intravenous administration of the potent bisphosphonate zoledronate caused substantial increases in BMD and suppression of bone turnover markers that were of similar magnitude to the effects of annual zoledronate in post-menopausal women, and to the effects of daily alendronate in men. It is likely that these effects on BMD would translate into efficacy in fracture prevention. The data from our other studies, and the current absence of definitive evidence of increased fracture risk in HIV-infected patients, suggest that routine administration of zoledronate to HIV-infected men is not appropriate. However, in those HIV-infected men who have experienced a fragility fracture or have osteoporosis and are at risk of such fractures, annual zoledronate provides an effective and convenient treatment option.

Taken together, the results of our cross-sectional and longitudinal studies, and the results of the control arm of our intervention study suggest that osteoporosis is not a significant health issue for HIV-infected men treated with HAART, and that routine monitoring of BMD is not necessary. It is important that previously published data on BMD in HIV-infected patients, in which the impact of body weight on BMD was not taken into account, are re-analysed to incorporate adjustments for body weight differences in HIV-infected patients compared to healthy controls.

There are many options for future research in this field. We plan to review previous studies that have reported BMD in HIV-infected patients and controls to determine whether there are similar between-groups differences in body weight to the difference we observed in Chapter 2, and whether adjusting for any such differences would account for the reported low BMD in previous studies of HIV-infected people. Another area of interest is the duration of effect of zoledronate. We plan to follow the participants in our study in an open-label observational extension to determine whether the effect of zoledronate on bone turnover and BMD may last longer than 12 months as is suggested by our results in Chapter 3.

## Chapter 5: The relationships between bone mineral density and parathyroid hormone, vitamin D, and body weight

### Introduction:

PTH and vitamin D and its metabolites are important hormones in the maintenance of serum calcium levels. Pathologically elevated levels of PTH or low levels of vitamin D metabolites have been associated with an increased risk of osteoporosis. Body weight is an important determinant of BMD in cross-sectional and prospective studies, and is an important risk factor for osteoporotic fractures. This chapter will briefly review the physiological roles of PTH and vitamin D, and the literature that links PTH, vitamin D, and body weight with BMD and secondary osteoporosis.

### PTH:

PTH is a hormone synthesised by the chief cells of the parathyroid gland that plays a central role in the regulation of calcium homeostasis [9]. There are three factors that control secretion of PTH: the extracellular calcium concentration,  $1,25(\text{OH})_2\text{D}$ , and the extracellular phosphate concentration. The most important factor is the extracellular calcium concentration, the effects of which are mediated through the calcium-sensing receptor. Elevated extracellular calcium levels lead to a decrease in PTH secretion while reduced levels lead to increased PTH secretion.  $1,25(\text{OH})_2\text{D}$  inhibits the expression of the *PTH* gene and may directly reduce PTH secretion while phosphate stimulates the expression of the *PTH* gene.

PTH has a number of effects on its target tissues, all mediated by a G protein-coupled receptor [9]. In the kidney, PTH stimulates the resorption of calcium in the distal tubule, inhibits the resorption of phosphate in the proximal and distal tubules, and stimulates the conversion of  $25\text{OHD}$  to  $1,25(\text{OH})_2\text{D}$ , the most active form of vitamin D.  $1,25(\text{OH})_2\text{D}$  in turn, stimulates calcium absorption from the intestine, inhibits PTH secretion and stimulates bone resorption. The effects of PTH on bone are complex. Acutely, PTH stimulates osteoclast-mediated release of calcium and phosphate from bone, while chronic exposure to PTH leads to increased osteoclastogenesis and osteoclast activity. However, intermittent administration of exogenous PTH leads to increased osteoblast activity and bone formation.

In summary, a fall in the extracellular calcium levels produces a rapid rise in PTH secretion which, in turn, increases release of calcium from bone, increases calcium resorption in the kidney, and increases calcium absorption from the intestine. These actions all cause an increase in the serum calcium level. Conversely, increased extracellular calcium levels produce a rapid decrease in PTH secretion, which in turn decreases calcium release from bone, decreases calcium resorption in the kidney, and decreases calcium absorption in the gut. These actions all cause a decrease in the serum calcium level. Thus PTH is an important factor in calcium homeostasis.

### **The effects of PTH on BMD and fractures:**

The effect of PTH on BMD is best observed in pathological states of PTH excess or deficiency. PHPT is characterised by elevated levels of serum calcium in the presence of normal or elevated serum PTH levels [155]. Severe untreated PHPT leads to the development of osteitis fibrosa cystica with evidence of widespread bone resorption, bone cysts and a predisposition to fractures, but this symptomatic bone disease is now rarely seen in countries where the measurement of serum calcium is automated and widely available [6, 156].

In asymptomatic PHPT, there is also evidence of the effects of elevated PTH on bone. Bone histomorphometry studies have shown that bone turnover is increased by approximately 50%. Trabecular bone mass and architecture are preserved while there is increased cortical porosity and bone loss [157]. In keeping with the differential effects of PHPT on bone seen histologically, the effects of PHPT on BMD appear to be different at different sites. In cross-sectional studies, at the distal forearm, a predominantly cortical site, there is reduction in BMD whereas at the lumbar spine, a predominantly trabecular site, BMD is preserved. The proximal femur has a mixture of trabecular and cortical bone and BMD changes at that site are intermediate between the spine and forearm [6, 158, 159]. Longitudinal studies of mild asymptomatic PHPT have produced conflicting results. Some studies of shorter duration have shown accelerated bone loss [159, 160] whereas studies of longer duration have shown stable BMD [161, 162]. Following curative parathyroidectomy in patients with PHPT, there are substantial increases in BMD at all sites [159, 162-164]. BMD increased by 12% at the lumbar spine and 14% at the femoral neck but did not change at the radius over 10 years following curative parathyroidectomy in the largest study with the longest duration of follow-up published to date [162].

Secondary hyperparathyroidism is characterised by elevated PTH levels in response to a low extracellular calcium concentration. The elevated PTH levels are usually sufficient to normalise the serum calcium level. The most common causes are vitamin D deficiency or chronic renal disease. In the long term, secondary hyperparathyroidism can progress to tertiary hyperparathyroidism, in which the serum calcium level becomes elevated. Both secondary hyperparathyroidism due to vitamin D deficiency [7] and secondary or tertiary hyperparathyroidism due to renal disease [165, 166] can be associated with lower than expected BMD. When BMD is low in these conditions, correction of the hyperparathyroidism (by vitamin D supplementation or parathyroidectomy) leads to rapid gains in BMD [167-170].

While elevated levels of PTH lead to lower BMD, PTH deficiency is associated with increased BMD. Idiopathic hypoparathyroidism is a rare condition that occurs usually because of abnormal parathyroid gland development or parathyroid gland destruction by a variety of mechanisms. In all these situations PTH levels are low and, as a consequence, serum calcium levels are low. BMD in idiopathic hypoparathyroidism has been reported to be higher than controls at the lumbar spine and proximal femur but not the radius, and the increase in BMD was associated with the duration of disease [171-174].

Chronically elevated PTH levels are also associated with increased risk of fracture. In severe untreated PHPT with osteitis fibrosa cystica there is an increased predisposition to fractures [156]. Asymptomatic PHPT is associated with a 1.5-1.8 fold increase in fractures at any site and a 3-3.5 fold increase in vertebral fractures [175]. There is a high prevalence of secondary hyperparathyroidism in patients with hip fracture [176], and in patients with renal failure on dialysis there is a U-shaped relationship between PTH levels and the risk of fracture [177].

In contrast to pathological states of PTH excess, intermittent pharmacological administration of PTH produces substantial increases in trabecular BMD, less impressive improvements in cortical BMD [178, 179] and prevents vertebral and non-vertebral fractures [178]. However, in pathological states of PTH excess, levels of PTH are chronically elevated which produces different effects on bone than intermittent PTH excess, both *in-vitro* as discussed previously and *in-vivo*.

In summary, there is consistent evidence from a number of different disorders that PTH has important clinical effects on bone density and fractures. Irrespective of the underlying cause,

people with chronically elevated levels of PTH tend to have lower BMD and higher rates of fractures than comparable healthy controls with normal PTH levels.

### **Vitamin D:**

Vitamin D is a hormone that is synthesised in the skin in response to UV-B radiation [8, 180]. In humans, a small amount is also obtained from dietary sources. However, the predominant source is the photolysis of the steroid precursor 7-dehydrocholesterol to previtamin D by UV-B radiation. Previtamin D undergoes a membrane-enhanced, temperature-dependent, isomerisation to vitamin D. Newly formed vitamin D binds to DBP and is transported to the liver where it is hydroxylated to 25OHD. 25OHD is further hydroxylated in the kidney to 1,25(OH)<sub>2</sub>D, the most active metabolite of vitamin D.

The main function of vitamin D, in combination with PTH, is to maintain stable serum calcium levels [8, 180]. Vitamin D has a number of different effects on target tissues mediated by active vitamin D metabolites binding to the vitamin D receptor in the target tissue. In the intestine, 1,25(OH)<sub>2</sub>D induces the expression of a number of calcium binding proteins that facilitate the transfer of calcium from the gut lumen to the circulation, leading to increased intestinal calcium absorption. In bone, 1,25(OH)<sub>2</sub>D promotes osteoclastogenesis leading to the release of calcium and phosphate into the serum. 1,25(OH)<sub>2</sub>D induces the expression of RANKL expression in osteoblasts. Osteoclast precursors expressing RANK recognise RANKL through cell-to-cell interaction with osteoblasts, and differentiate into mature osteoclasts [181]. 1,25(OH)<sub>2</sub>D has a number of additional effects on osteoblasts. These effects include inducing production of OPG in mature osteoblasts [182], promoting osteoblast differentiation, stimulating mineralisation, inhibiting osteoblast proliferation, and altering the synthesis and production of proteins, enzymes and growth factors [183]. In the parathyroid gland, 1,25(OH)<sub>2</sub>D decreases the expression of the *PTH* gene and decreases PTH synthesis. In combination, these actions of vitamin D play a key role in calcium homeostasis. Thus, low vitamin D status leads to decreased intestinal calcium absorption, which in turn leads to low serum calcium levels. The homeostatic response to low serum calcium levels is increased 1,25(OH)<sub>2</sub>D levels (mediated via increased PTH, i.e. secondary hyperparathyroidism) which in turn leads to increased intestinal absorption of calcium, the release of calcium from bone, and a rise in the serum calcium level. Conversely, vitamin D intoxication leads to high serum calcium levels. The homeostatic response to high serum calcium levels is lowered 1,25(OH)<sub>2</sub>D levels (mediated via lowered PTH) which in turn leads

to reduced intestinal calcium absorption, increased uptake of calcium into bone, and a lowering of the serum calcium.

Vitamin D has a number of non-calcaemic actions. The vitamin D receptor is expressed in most tissues in the body, and, *in-vitro*, the binding of 1,25(OH)<sub>2</sub>D to its receptor has numerous effects including inhibition of cellular proliferation, induction of terminal differentiation of cells, stimulation of insulin production, and modulation of the immune response. It is not known what the exact role of these functions are *in-vivo*. Consistent with the multitude of effects seen *in-vitro*, vitamin D deficiency has been associated in observational studies with a number of non-skeletal disorders including myopathy, many types of cancer, multiple sclerosis, hypertension, diabetes mellitus, ischaemic heart disease, congestive heart failure and rheumatoid arthritis [184].

### **The effects of vitamin D on bone turnover, BMD and fractures:**

The best estimate of the vitamin D status of an individual is measurement of serum 25OHD levels [8, 180]. Estimates of the threshold level of serum 25OHD above which vitamin D stores are considered adequate vary widely, from 25 nmol/L to 100 nmol/L [185, 186]. A commonly used definition is that levels of 25OHD less than 50 nmol/L are consistent with vitamin D insufficiency, and levels of 25OHD less than 25 nmol/L are consistent with vitamin D deficiency [7]. There is evidence from a number of different sources that vitamin D insufficiency or deficiency has deleterious effects on bone.

Low levels of 25OHD are associated with increased markers of bone turnover such as ALP, bsALP, OC, urine NTx, urine DPD and serum CTx [7, 187-189]. This is most likely mediated through PTH: low 25OHD levels may lead to a reduction in intestinal calcium absorption, stimulating an increase in circulating levels of PTH, which in turn leads to increased bone turnover [7].

BMD has been associated with vitamin D status in some [190-192] but not all [193-195] cross-sectional studies. Investigators in the MORE study [191] showed that a 25OHD level in the lowest tertile (less than 25 nmol/L) was associated with a 4% lower trochanteric BMD, although there was no effect on lumbar spine or femoral neck BMD. A cross-sectional study performed in 330 elderly women in Amsterdam, showed a positive correlation between serum 25OHD and BMD of the hip [190], but with a threshold of effect, such that when 25OHD was

greater than 30 nmol/L, the relationship was no longer significant. The largest published cross-sectional study reported data from 13,432 participants in NHANES III [192]. In that study there was a positive relationship between 25OHD and BMD in all subgroups of participants up to levels of 25OHD of 90-100 nmol/L.

There are few large prospective epidemiological studies that have examined the association between baseline vitamin D status or 25OHD levels and subsequent risk of fracture. In those studies that have reported these data, there has not been a consistent relationship between 25OHD levels and fracture risk [196-200]. This may be in part due to the small number of fractures observed, and the small number of participants with low 25OHD levels. However, seasonal variations in fractures have been reported that appear to closely parallel the seasonal variation in 25OHD levels [189], and numerous cross-sectional studies have reported low 25OHD levels in patients who have sustained osteoporotic fractures [201].

Intervention studies using vitamin D (with or without calcium supplementation) have reported small increases in BMD with vitamin D supplementation [7, 202, 203], although studies of institutionalised patients with low 25OHD levels have reported greater responses [170, 204]. Chapuy et al. randomised 3270 healthy, post-menopausal women living in nursing homes to supplementation with 1.2 g/day calcium and 800 IU/day cholecalciferol or placebo [170]. After 18 months, BMD of the proximal femur had increased by 2.7 percent in the group treated with vitamin D and calcium, but decreased by 4.6 percent in the placebo group ( $P < 0.001$ ). The same group repeated this study in a two-year trial of 583 healthy, institutionalised women randomised to supplementation with 1.2 g/day calcium and 800 IU/day cholecalciferol or placebo [204]. Femoral neck BMD increased by 0.3%/year in the group treated with calcium and vitamin D, but decreased by 2.4%/year in the placebo group ( $P = 0.09$ ). Dawson-Hughes et al. randomised 176 men and 213 women over 65 years of age living in the community to supplementation with 500 mg/day calcium and 700 IU/day cholecalciferol or placebo for three years [202]. At the femoral neck, BMD increased by 0.5% in the calcium and vitamin D group but decreased by 0.7% in the placebo group ( $P = 0.02$ ). At the lumbar spine, BMD increased by 2.1% in the calcium and vitamin D group, and by 1.2% in the placebo group ( $P = 0.04$ ).

Data on fracture incidence following vitamin D supplementation are conflicting. In the landmark study by Chapuy et al. there was a 43 percent reduction in hip fractures ( $P = 0.04$ ), and a 32 percent reduction in non-vertebral fractures ( $P = 0.015$ ) in the women treated with



calcium and vitamin D after 18 months. The difference in fracture rates between the groups persisted and, after 36 months of supplementation, there was a 29% reduction in hip fractures and a 24% reduction in non-vertebral fractures in the women treated with calcium and vitamin D [205]. While the study by Dawson-Hughes et al. was not powered to detect differences between groups in numbers of fractures, of 37 subjects who had non-vertebral fractures, 26 were in the placebo group and 11 were in the calcium and vitamin D group ( $P= 0.02$ ) [202]. The data on fracture prevalence from these and other intervention studies using vitamin D supplementation have been summarised in two recent meta-analyses [203, 206]. A vitamin D dose of 700 to 800 IU/day reduced the relative risk of hip fracture by 26% (95% CI 12% to 39%), and the relative risk of a non-vertebral fracture by 23% (95% CI 13% to 32%), but there were insufficient data to draw conclusions on the relative risk of vertebral fractures. In contrast there were no significant benefits on fracture prevention with 400 IU/day vitamin D.

Subsequent to these meta-analyses, three large randomised controlled trials on the effects of vitamin D supplementation have been published. In the RECORD study, 5292 patients with a history of low-trauma fracture were randomised to supplementation with 1000 mg/day calcium, 800 IU/day cholecalciferol, calcium plus cholecalciferol, or placebo [207]. There were no differences in fracture rates between the groups. However, interpretation of the results is limited by poor compliance (fewer than 50% of participants took tablets for more than 80% of the study), and the lack of detailed assessment of vitamin D status at baseline (25OHD levels were only measured in approximately 1% of participants). Porthouse et al. randomised 3314 older women with risk factors for fractures to supplementation with 1000 mg/day calcium and 800 IU/day cholecalciferol or to a leaflet describing how to consume adequate calcium and vitamin D from dietary sources [208]. While there were no difference in fracture rates between the groups, the study design and lack of assessment of 25OHD levels prevents conclusions being drawn on the efficacy of vitamin D supplements. The WHI study reported on the effect of supplementation with 1000 mg/day calcium and 400 IU/day cholecalciferol compared to placebo in 36,282 post-menopausal women [200]. There was no difference in fracture rates between groups, but there was a small increase in hip BMD, but no difference at the spine or total body, with calcium and vitamin D in a subgroup of 2400 women who underwent BMD testing. Again, interpretation of the study is difficult because of the high use of concomitant hormone replacement therapy, the assessment of 25OHD levels in only approximately 5% of study participants, the low baseline risk of

fracture in the participants, and the study design that permitted personal vitamin D supplementation up to 600 IU/day.

A Cochrane review carried out prior to publication of the WHI study reported differing findings to the earlier meta-analyses [209]. There was no reduction in hip or total fractures with vitamin D supplementation, although subgroup analyses by dose of vitamin D were not carried out. However, there was a 19% (95% CI 4% to 32%) reduction in the relative risk of hip fractures and a 13% (95% CI 3% to 22%) reduction in the relative risk of non-vertebral fractures with combined calcium and vitamin D supplementation, but these benefits appeared restricted to elderly institutionalised women.

In summary, there is consistent evidence that vitamin D supplementation of at least 700 IU/day (in combination with calcium supplementation) in people at high risk of fracture with low 25OHD levels increases BMD and reduces fracture risk. A beneficial effect of vitamin D supplements has not been established in groups at lower risk of fracture or with higher 25OHD levels, or at lower doses of vitamin D supplementation.

Finally, in vitamin D deficiency where 25OHD levels are very low, osteomalacia may occur. Osteomalacia is characterised by increased unmineralised osteoid and may lead to bone pain, low BMD, and fracture. Studies have reported high prevalence of osteomalacia in histological analyses of bone specimens from patients with hip fractures [7].

In summary, low vitamin D status is associated with secondary hyperparathyroidism, increased bone turnover, low BMD and increased risk of fracture. Overt vitamin D deficiency can cause osteomalacia, and both low 25OHD levels and osteomalacia are relatively common findings in patients with hip fractures. Intervention studies in patients at high risk of osteoporotic fracture with low 25OHD levels have shown an increase in BMD and reduction in fracture risk with doses of vitamin D greater than 700 IU/day.

### **The relationship between PTH and Vitamin D:**

As discussed previously, vitamin D insufficiency can lead to reduced intestinal calcium absorption. Secondary hyperparathyroidism may then occur as a homeostatic response to maintain serum calcium levels. In cross-sectional studies, 25OHD levels are inversely associated with PTH levels [191, 210, 211]. Chapuy et al. reported that in healthy adult men and women PTH levels were stable above a 25OHD level of 78 nmol/L, while PTH levels

increased exponentially below this level [210]. Dawson-Hughes et al. reported similar findings in elderly men and women but that the PTH levels increased slowly below a 25OHD level of 110 nmol/L [211]. Lips et al. reported that in post-menopausal women a 25OHD level less than 25 nmol/L was associated with a 30% increase in PTH levels compared to women with a 25OHD level more than 50 nmol/L, while women with 25OHD levels between 25 and 50 nmol/L had a 15% increase in PTH levels [191]. Cross-sectional analyses of 25OHD and PTH levels can be flawed. Some researchers have used Pearson correlation analysis to assess the linear relationship between 25OHD and PTH, and typically such studies report a correlation coefficient between PTH and 25OHD of -0.20 to -0.30 [191, 212], however most other researchers have found non-linear relationships between 25OHD and PTH [210, 211]. There are pronounced seasonal variations in 25OHD and PTH levels in countries distant from the equator [213]. The changes in PTH levels lag behind the changes in 25OHD levels by approximately one month [189], a time approximately equivalent to the serum half-life of 25OHD [214]. Cross-sectional analyses do not take this lag into account, and therefore compare values of 25OHD and PTH from different points in their seasonal cycles.

A number of intervention studies have shown that vitamin D supplementation in people with low 25OHD levels leads to a fall in serum PTH levels. Malabanan et al. reported that vitamin D supplementation with 50,000 IU weekly for eight weeks led to 35% decrease in PTH levels in people with 25OHD levels of 27.5-39.9 nmol/L, and a 26% decrease in PTH levels in people with 25OHD levels of 40-49.9 nmol/L, but no change in PTH levels in people with 25OHD levels greater than 50 nmol/L [215]. Lips et al. reported similar results from the MORE study with 400-600 IU/day of vitamin D supplementation [191].

In summary, low 25OHD levels are consistently associated with increased PTH levels. This inverse relationship is non-linear. There is conflicting evidence as to whether there is a threshold level of 25OHD above which PTH levels do not decrease further, and, if such a threshold exists for 25OHD, at what serum level this occurs.

## **The effects of body weight and body composition on bone turnover, BMD and fractures:**

Low body weight is associated with increased bone turnover. Body weight or BMI are inversely related to markers of bone formation and resorption in cross-sectional studies [216,

217]. Changes in weight also influence bone turnover markers. Short term weight loss leads to increased bone turnover, with increases in both formation and resorption markers [218].

Body weight is a major determinant of BMD. Cross-sectional studies have shown consistent, moderately strong, positive correlations ( $r= 0.3-0.6$ ) between body weight and BMD in men and women throughout adulthood, and also in children [10]. Prospective studies have shown that changes in BMD over time are related to both baseline body weight and the change in body weight over time [140, 219]. These findings have been confirmed in very large epidemiological studies [217, 220].

Body weight is also a major determinant of the risk of osteoporotic fracture [10]. Many prospective studies have reported that baseline body weight and change in body weight over time are related to the subsequent risk of fracture. In the Study of Osteoporotic Fractures, women in the lowest weight quartile had twice the number of hip fractures than women in the highest quartile [221]. Similarly, women in the lowest quintile of weight gained since 25 years of age had 15 times the rate of fracture compared to women in the highest quintile [222]. In the EPIDOS study, women sustaining a hip fracture over a two year period were 2.4 kg lighter than women who did not sustain a hip fracture [223]. In the European Vertebral Osteoporosis Study, in women the risk of vertebral fracture was inversely related to the quintile of current body weight, current BMI, and weight gain since 25 years of age. In contrast, in men, only those in the lowest quintile for these variables had an increased risk of fracture [224].

The effects of the soft tissue components of body weight— fat mass and lean mass— on bone density and fracture risk have been reported. In pre-menopausal and post-menopausal women, fat mass and changes in fat mass over time are major determinants of BMD in cross-sectional and longitudinal studies. In contrast lean mass is not related to BMD after adjustment for skeletal size [140, 219, 225]. In men, neither fat mass nor lean mass were related to BMD after adjustment for skeletal size [225]. In the Study of Osteoporotic Fractures, those women in the lowest quartile of both fat and lean mass (measured using electrical bioimpedance) had the highest rates of hip fracture [221]. In the EPIDOS study (where body composition was measured using DXA), there was no relationship between baseline lean mass and the subsequent risk of fracture, whereas the relative risk of fracture per decrease in standard deviation of fat mass was 1.4 [223].

In summary, body weight is a major determinant of BMD and fracture risk. Fat mass is consistently associated with BMD and fracture risk in pre- and post-menopausal women but not men, whereas lean mass is not consistently associated with BMD or fracture risk in men or women.

### **Conclusion:**

PTH, vitamin D, and body weight all have important effects on BMD. Elevated PTH levels, low 25OHD levels, and low body weight are important risk factors for low BMD and the risk of fracture. Secondary osteoporosis can be caused by exposure to these variables. While PTH and vitamin D levels are closely interrelated, there is also evidence to suggest that body weight may be related to both PTH and vitamin D. The purpose of the subsequent chapters in this second part of the thesis is to explore the relationships between PTH and body weight, and between vitamin D metabolites and body weight, and to explore the determinants of PTH and vitamin D metabolites in a variety of population groups.

## **Chapter 6: The relationship between primary hyperparathyroidism and body weight**

### **Introduction:**

PHPT is one of the most common endocrine conditions, particularly in post-menopausal women. The prevalence of PHPT has been estimated at 3/1000 in the general population, and as high as 21/1000 in post-menopausal women [226]. The incidence in Rochester, Minnesota, is estimated to be 20/100,000/year [227]. Frequently, it appears to be asymptomatic, and there is uncertainty as to the optimal management of this form of the disease. Most longitudinal studies indicate that the hypercalcaemia is not progressive, and that renal function remains stable [162, 228, 229]. Such data suggest that it is reasonable to leave asymptomatic individuals untreated [162]. However, there is epidemiological evidence, primarily from European studies, that suggests that PHPT is associated with increased incidences of hypertension, insulin resistance, dyslipidaemia, cardiovascular disease and malignancy [230-238]. These considerations would argue in favour of intervention, even in asymptomatic individuals, in the hope of reducing morbidity from these conditions. While the recent NIH consensus conference guidelines [155] do not support such an approach, a number of editorials have argued that parathyroidectomy should be performed for almost all patients with PHPT [239-241].

In 1994, our group reported that body weight was increased in a cohort of post-menopausal women with asymptomatic PHPT [158]. Subsequently, we reported that the increase in body weight appeared to antedate the development of the hypercalcaemia [242]. This suggests that the increase in weight is not secondary to PHPT, and therefore is unlikely to be affected by parathyroid surgery. Since increased body weight is known to be associated with hypertension, insulin resistance, dyslipidaemia and cardiovascular disease [243], it is possible that increased body weight accounts for these other associations of PHPT. If this is the case, surgical correction of the parathyroid abnormality is unlikely to impact on the frequency of these other conditions. Indeed, what data are available suggest that parathyroidectomy does not influence hypertension, dyslipidaemia, or the incidence of cardiovascular disease [233, 234, 236, 237, 244-247].

Clearly, the possible association of PHPT with increased body weight is central to an understanding not only of the aetiology of this condition but also of its morbidity and optimal

treatment. Since this question does not appear to have been specifically revisited since the time of the earlier publications from our group, we have meta-analysed all the published studies which report body weight in subjects with PHPT and in age- and sex-comparable healthy controls.

## **Methods:**

### ***Study Selection:***

MEDLINE was searched from 1975 to 2003 using the term “primary hyperparathyroidism”. To be eligible for consideration, a study must have been published in English, have had a control group, and have presented data on body weight or BMI for both groups. Fifty-nine studies meeting these criteria were identified. Further specific exclusion criteria were:

(1) groups not comparable for mean age (within seven years) or gender (male:female ratio within 15%); (2) groups matched for weight, BMI, or body size; (3) groups matched for other variables which are likely to be related to body weight (blood pressure, glucose tolerance); (4) studies of subjects post-parathyroidectomy; and (5) studies where the control group was selected from a population with comorbidities likely to significantly impact on body weight (such as diabetes mellitus, osteoporosis, vascular disease, malignancy). Where the same cohort was described in more than one publication, only the largest study conforming to the inclusion and exclusion criteria was included. Each of the 59 studies identified was independently assessed by my two supervisors and I, and a consensus was reached regarding the suitability of each study without knowledge of the body weight data.

### ***Statistics:***

Data were analysed using Review Manager (RevMan) version 4.2.3 for Windows (The Cochrane Collaboration, Oxford, England, 2003). Weighted mean differences in weight and BMI were calculated for all eligible studies using a fixed effects model. A global analysis was performed using the standardised mean differences for body weight and BMI. In the presence of significant heterogeneity ( $P < 0.10$ ), a random effects model was also run in an attempt to incorporate the differences between studies into the analysis. Funnel plots for each model were inspected. All tests were two-tailed; a P value of  $< 0.05$  was considered statistically significant and 95% CIs are presented.

## **Results:**

Of the 59 studies identified, 15 were excluded because of previous or subsequent publication of the study group; nine because of matching for weight, BMI or body size; eight because the control groups were not comparable in age or gender distribution; four because of control group comorbidities; three because of matching for blood pressure; and two because they were carried out after parathyroid surgery. Thus, 18 studies were eligible for inclusion in the review: 14 studies (Table 6.1) presenting body weight [158, 164, 172, 235, 248-257] and four studies [258-261] presenting BMI (Table 6.2). Dalberg et al. [252] presented data as median  $\pm$  range. These data were not included in the final analysis because of the lack of statistical compatibility with the other data. Grey [253] presented one group of pre-menopausal and a separate group of post-menopausal subjects each with a matched control group. These two groups were analysed separately. Hagag et al. [261] published data on two groups of patients with PHPT. These data were pooled and compared to the solitary control group. Thus the final analysis contained 17 studies that collectively reported data on 617 patients with PHPT and 1248 controls.

Subjects with PHPT were on average 3.34 kg (95% CI 1.97 to 4.71,  $P < 0.00001$ ) heavier than controls in the 13 studies presenting body weight (Figure 6.1). In the study excluded because it reported group weights as median  $\pm$  range, the median weight in subjects with PHPT was 7 kg greater than in the controls, a result consistent with the other studies. In three of the four studies presenting BMI, subjects with PHPT had an increased BMI compared to the controls. However when analysed using a random effects model (necessitated by significant heterogeneity,  $P = 0.04$ , between studies), the difference in BMI between groups was  $1.13 \text{ kg/m}^2$ , a result that fails to reach statistical significance ( $P = 0.12$ , 95% CI -0.29 to 2.55) (Figure 6.2). While BMI is related to weight, it cannot be converted to weight without knowledge of the height of the individual subjects. A measure of overall effect can however be obtained by pooling data from the studies reporting weight and BMI and applying a standard mean difference analysis. This analysis demonstrated that body weight or BMI was on average 0.3 SD (95% CI 0.19 to 0.40,  $P < 0.00001$ ) higher in the subjects with PHPT than the controls (Figure 6.3). This is equivalent to 3.1 kg in terms of weight and  $1.1 \text{ kg/m}^2$  in terms of BMI.



**Table 6.1: Studies of patients with PHPT and eucalcaemic controls presenting data on body weight**

Study	Patients with Primary PHPT							Controls						
	N	Sex (%F)	Post menopausal	Age (Yr)	Weight (kg)	Serum Calcium (mmol/L) <sup>a</sup>	Serum PTH (pmol/L) <sup>b</sup>	N	Sex (%F)	Post menopausal	Age (Yr)	Weight (kg)	Serum Calcium (mmol/L) <sup>a</sup>	Serum PTH (pmol/L) <sup>b</sup>
Camozzi [257]	51	78	27.5	55.9 (14.1)	67.1 (13.2)	2.75 (0.15)	13.2 (5.5)	58	78	71.1	53.3 (12.5)	65.4 (13.7)		
Christiansen [164]	25	80	40	53 (13)	74 (16)	2.71 (0.16)	12.5 (6.3)	25	80	40	53 (13)	71 (13)		
Cortet [254]	26	77		54 (17)	77.9 (19.2)	2.72 (0.15)	10.2 (4.7)	35	77		54 (18)	67.8 (12.7)		
Dalberg [252]	44	73		61 (52-70) <sup>c</sup>	75 (60-83) <sup>c</sup>	2.78 (2.69-2.92) <sup>c</sup>	9.1 (8.1-11.3) <sup>c</sup>	23	87		55 (51-67) <sup>c</sup>	68 (63-70) <sup>c</sup>	2.35 (2.27-2.39) <sup>c</sup>	
Duan [172]	25	100	100	68.7 (1.6) <sup>d</sup>	69.7 (2.5) <sup>d</sup>			405	100	100	63.8 (0.4) <sup>d</sup>	65.5 (0.6) <sup>d</sup>		
Gonnelli [255]	22	91		61.3 (10.9)	63.2 (10.4)	1.51 (0.1) <sup>e</sup>	24 (10.6)	22	91		61.1 (10.7)	62.7 (9.3)	1.2 (0.04) <sup>e</sup>	3.6 (1)
Grey [158]	41	100	100	67 (1.2) <sup>d</sup>	75.5 (2.3) <sup>d</sup>	1.42 (0.08) <sup>e</sup>	9.4 (4.7)	43	100	100	65.7 (0.52) <sup>d</sup>	66.3 (1.5) <sup>d</sup>		
Grey [253]	19	100	0	39.4 (8.7)	68.4 (11)			34	100	0	37.4 (6.1)	61.2 (8.7)		
Grey [253]	36	100	100	59.3 (4.2)	71 (8.8)			122	100	100	58 (5)	65.6 (9)		
Ingle [256]	25	100	100	61.1 (9.5)	69.5 (14.1)	2.84 (2.62-3) <sup>f</sup>	11.4 (3.6-10.9) <sup>f</sup>	95	100	100	67 (6.3)	67.1 (10.4)		
Joborn [249]	18	89		62 (12)	68 (13)	2.77 (0.15)	1.02 (0.21) <sup>g</sup>	20	100		60 (8)	66 (10)		
Lundgren [235]	102	100	100	66.4 (5.83)	70.2 (13.23)	2.6 (0.14)	6.4 (3.7)	102	100	100	66.6 (5.73)	68.8 (10.73)	2.37 (0.08)	3.3 (1.1)
McDermott [250]	43	100	100	63 (2) <sup>d</sup>	70 (2) <sup>d</sup>	2.68 (0.03) <sup>d</sup>	2.8 (0.3) <sup>d,h</sup>	84	100	100	60 (1) <sup>d</sup>	66 (1) <sup>d</sup>		
Prager [248]	15	47		46.3 (2.3) <sup>d</sup>	66.3 (2.6) <sup>d</sup>	2.93 (0.1) <sup>d</sup>	9.5 (2.1) <sup>d</sup>	20	50		43.5 (2.5) <sup>d</sup>	67.8 (1.4) <sup>d</sup>	2.3 (0.05)	3.4 (0.2)
Roland [251]	10	80		44 (15.8)	54.7 (10.4)	3.05 (0.27)		10	70		39 (12.87)	58.5 (5.85)	2.34 (0.12)	

All data are presented as mean (SD) or percent unless stated

<sup>a</sup> normal range varies between laboratories but typical normal range is 2.10-2.55 mmol/L

<sup>b</sup> normal range varies between laboratories but typical normal range is 1.0-7.0 pmol/L

<sup>c</sup> data are presented as median (range), data not included in final analysis because of statistical incompatibility

<sup>d</sup> data are presented as mean (SEM)

<sup>e</sup> ionised calcium, normal range 1.12-1.23 mmol/L

<sup>f</sup> data are presented as mean (range)

<sup>g</sup> units arbitrary u/L normal range 0.4-1.08

<sup>h</sup> units pg/mL normal range 0-1.5

**Table 6.2: Studies of patients with PHPT and eucalcaemic controls presenting data on BMI**

Study	Patients with Primary PHPT						Controls					
	N	Sex (%F)	Age (Yr)	BMI (kg/m <sup>2</sup> )	Serum Calcium (mmol/L) <sup>a</sup>	Serum PTH (pmol/L) <sup>b</sup>	N	Sex (%F)	Age (Yr)	BMI (kg/m <sup>2</sup> )	Serum Calcium (mmol/L) <sup>a</sup>	Serum PTH (pmol/L) <sup>b</sup>
Elvius [258]	48	100	58 (3)	26 (4)			46	100	60 (3)	25 (4)		
Hagag [261]	13	100	59 (10)	30 (6)	2.75 (0.13)	14.9 (8.7)	25	100	56 (12)	25 (4)	2.38 (0.2)	3.4 (1.5)
Hagag [261]	29	100	63 (11)	28 (3)	2.75 (0.08)	11.5 (4.9)	25	100	56 (12)	25 (4)	2.38 (0.2)	3.4 (1.5)
Jorde [259]	39	72	64.6 (10.3)	25.7 (3.9)	2.56 (0.12)	12.4 (5.6)	72	75	65.6 (9.4)	25.3 (3.5)	2.26 (0.1)	4.7 (1.6)
Nilsson [260]	30	77	64 (13)	26.1 (0.7) <sup>c</sup>	2.97 (0.04) <sup>c</sup>	15.7 (1.7) <sup>c</sup>	30	77	64 (12)	26.2 (0.6) <sup>c</sup>	2.37 (0.02) <sup>c</sup>	4.1 (0.4) <sup>c</sup>

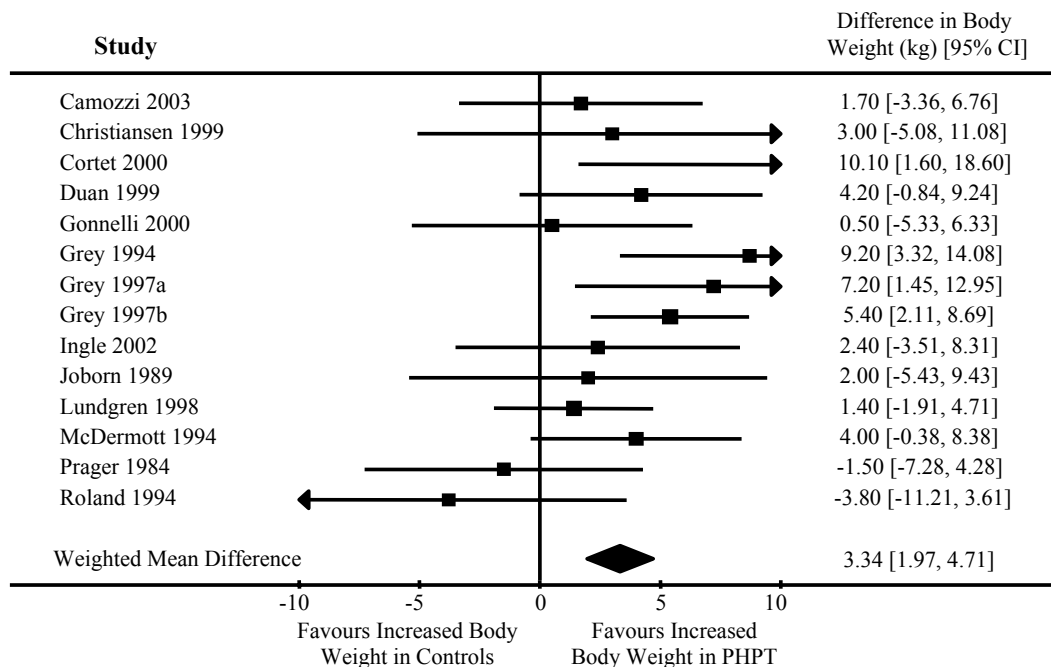
All data are presented as mean (SD) unless stated.

<sup>a</sup> normal range varies between laboratories but typical normal range is 2.1-2.55 mmol/L

<sup>b</sup> normal range varies between laboratories but typical normal range is 1.0-7.0 pmol/L

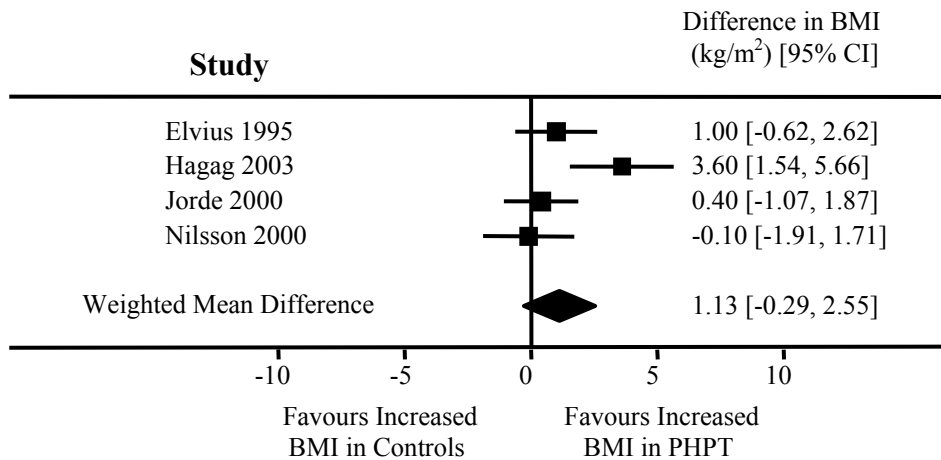
<sup>c</sup> data are presented as mean (SEM)

**Figure 6.1: Weighted mean difference in body weight in studies of PHPT and eucalcaemic controls that present data on body weight**



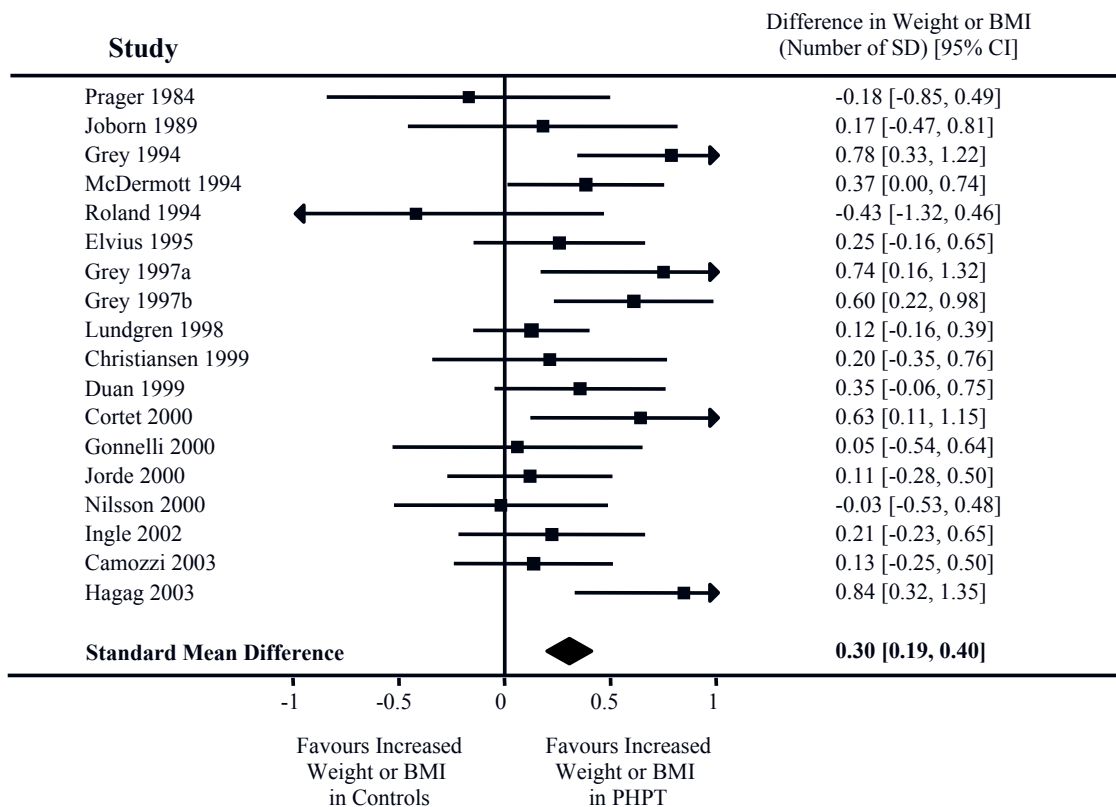
P for heterogeneity between trials = 0.13. The lines show 95% CI for each study, and the diamond shows 95% CI for the pooled analysis. P for weighted mean difference < 0.00001.

**Figure 6.2: Weighted mean difference in BMI in studies of PHPT and eucalcaemic controls that present data on BMI**



P for heterogeneity between trials = 0.04. The lines show 95% CI for each study, and the diamond shows 95% CI for the pooled analysis. P for weighted mean difference = 0.12.

**Figure 6.3: Standard mean difference in studies of PHPT and eucalcaemic controls that present data on weight or BMI**



The lines show 95% CI for each study, and the diamond shows 95% CI for the pooled analysis. P for standard mean difference < 0.00001

The finding of increased body weight in association with PHPT was consistent across the studies. Only three studies did not report higher body weight or BMI in the subjects with PHPT. These three studies had features suggesting that their subjects were not representative of patients with asymptomatic PHPT and mild hypercalcaemia. In all three studies, the means for serum calcium were higher than in the remainder of the studies (2.93-3.05 mmol/L compared to 2.6-2.83 mmol/L). In one of the studies, all subjects had osteitis fibrosa cystica or renal stones [248]. Two of the studies were in patients going forward for parathyroid surgery [251, 260], and two were small (15 and 10 subjects respectively) and had younger subjects (mean age 46.3 and 44 years) than the remainder [248, 251].

The degree of hypercalcaemia appeared to have an important influence on body weight. In the three studies where the mean serum calcium was at least 2.93 mmol/L, the groups with PHPT weighed less than the control groups. In the remaining studies where the mean serum calcium was less than 2.83 mmol/L, the groups with PHPT were heavier than the controls, and the difference in body weight between groups did not correlate with mean serum calcium.

There was a predominance of females in the study groups (563 of 617 participants), reflecting the well-known difference in prevalence of PHPT between genders. Where menopausal status was given, 313 of 351 women were recorded as post-menopausal; however in 212 women the menopausal status was not recorded. When subjects were analysed by categories (post-menopausal, pre-menopausal, menopausal status not recorded, or male), subjects with PHPT were consistently heavier than controls across all groups by amounts similar to the overall pooled result. The weight or BMI (95% CI) increases for subjects with PHPT for each category were: post-menopausal women 3.97 kg (2.33 to 5.61),  $N = 313$ ,  $P < 0.00001$ ; pre-menopausal women 4.44 kg (0.76 to 8.13),  $N = 48$ ,  $P = 0.02$ ; women menopausal status not recorded 2.07 kg/m<sup>2</sup> (0.53 to 3.60),  $N = 130$ ,  $P = 0.008$ ; men 2.82 kg (-5.74 to 11.37),  $N = 16$ ,  $P = 0.52$ .

Only two studies reported data on body composition [158, 164]. In both studies the majority of the weight difference (7.2 of 9.2 kg, and 2.6 of 4.5 kg respectively) was attributable to increased fat mass.

## **Discussion:**

This meta-analysis confirms the earlier findings by our group that subjects with PHPT are heavier than eucalcaemic control subjects. Most of the individual studies were small, with

only a modest weight difference, and in 10 studies the between-groups difference in body weight did not reach statistical significance. This explains why the observation that body weight is increased in PHPT has not been generally recognised. However, the results are consistent across 13 studies reporting body weight and four studies reporting BMI. The differences between the results of the three studies that did not report higher body weight or BMI in the group with PHPT and the remainder raise the possibility that the anorectic effects of moderate hypercalcaemia [262] may negate the weight differences observed in mild PHPT. Alternatively, the pathogenesis of PHPT in young adults may be different from that in older subjects, paralleling the marked differences in the epidemiology of the condition between these groups [263, 264].

The increases in weight and BMI observed here in subjects with PHPT are likely to be of clinical significance. Data from the Nurses Health Study and the Health Professionals Follow-up study of more than 120,000 men and women followed for 10 years [265] predict that an increased BMI of 1.1 kg/m<sup>2</sup> in women would increase the risk of diabetes mellitus by 155%, gallstones by 20%, hypertension by 15%, and heart disease by 6%. The predicted effects in males are of similar magnitude, except that the predicted risks of hypertension and heart disease are almost twice those of women. Similarly, data from the Asia Pacific Cohort Studies Collaboration, which includes data from more than 300,000 men and women contributing over 2 million person years of follow-up [266], predict that an increase in BMI of 1.1 kg/m<sup>2</sup> would increase the risk of ischemic heart disease by 6%. Data from the INTERSALT trial [267] predict that a 3.1 kg weight difference would be associated with an increase in blood pressure of 0.9/0.7 mmHg. Using data from the Cancer Prevention Study II [268], a prospective study in the United States of more than one million people, an increased BMI of 1.1 kg/m<sup>2</sup> would predict an increased all-cause mortality of 6% in women and 10% in men.

A number of longitudinal studies of subjects with PHPT have reported an increased risk of cardiovascular disease and/or mortality [228, 232, 233, 236-238, 244, 245, 247, 269-271], although not all studies have confirmed these risks [272, 273]. None of these studies has reported or adjusted for body weight. Reported standardised mortality rates in subjects with PHPT range from 1.2 to 1.7, while relative risks for cardiovascular disease range from 1.7 to 2.5. Increased body weight may contribute to the increased risk of cardiovascular disease in PHPT, although it may not account entirely for the reported increase in risk. We have previously published data showing that increased body weight antedates the diagnosis of

PHPT [242] and was in fact present at all ages throughout the adult lives of patients with PHPT. The longer lifetime exposure to increased body weight in patients with PHPT may exacerbate the risks of complications of obesity when compared to the participants in epidemiological studies in which the average duration of follow-up was only 10 years. Other authors have suggested that the increased cardiovascular risk may be due to vascular and cardiac abnormalities such as calcifications, in part mediated by hypercalcaemia, but the evidence for this is not compelling [274].

Increased body weight is associated with an increased risk of several important vascular risk factors: hypertension, hyperlipidaemia, and glucose intolerance. Each of these risk factors has also been associated with PHPT, providing a potential explanation for the increased risk of vascular disease in PHPT. Thus, increased blood pressure of 18/7 mmHg has been reported in subjects with PHPT [275], while hypertension may occur in 20-50% of such patients [236]. Although increased body weight cannot explain the magnitude of the observed difference in blood pressure, it could explain the increased prevalence of hypertension. The finding that hypertension does not improve after parathyroidectomy [234, 276, 277] suggests that hyperparathyroidism itself does not have a causal role. The data regarding dyslipidaemia in PHPT are conflicting. One study reported high triglycerides, low high-density lipoprotein cholesterol, and an atherogenic lipid profile in patients with PHPT, which were normalised by surgical cure [278], while others reported normal lipid levels with no change after parathyroidectomy [246, 279-281]. With regard to glucose intolerance, several studies have shown a 2-3 fold increased risk of diabetes in PHPT [231, 282, 283]. While insulin resistance is present on oral or intravenous glucose tolerance testing and improves after parathyroidectomy, there is little change in the fasting glucose or HbA1c [231, 246, 248, 284-286]. Thus, while hyperparathyroidism may cause insulin resistance, the increased body weight may explain the increased prevalence of diabetes and the lack of response to surgery.

Increased body weight has also been associated with gallstone disease and an increased risk of death from a variety of cancers, including those of the gastrointestinal tract, kidney, breast, and female reproductive tract [287]. Some, but not all, data suggest an increased prevalence of cholelithiasis in PHPT [288, 289]. Reports have detailed a 1.5-2 fold increase in the risk of cancer occurrence [230, 232], and increased mortality from cancer [238] in PHPT. A variety of cancers were implicated, including tumours of the gastrointestinal tract, kidney and breast.

Thus, increased body weight may contribute to the associations reported between several malignancies and PHPT.

A key question that arises from this study is the mechanism of the association between body weight and PHPT. The cause-and-effect relationship could operate in either direction. For instance, there is evidence that increased intracellular calcium concentrations in adipocytes cause insulin resistance and inhibit lipolysis [290], and PTH elevates intracellular calcium in many cell types [291]. It is also possible that PTH influences adipocyte differentiation, since adipocytes and osteoblasts share common precursor cells [125] and PTH acts directly on osteoblasts [292]. These observations would imply that increased fat mass is the main contributor to the increased body weight and is a consequence of PHPT, and therefore likely to be corrected by parathyroidectomy. To our knowledge, there is only one published study on the effects of surgical correction of PHPT on fat mass or body weight [164]. In that study, three years following parathyroidectomy there was no change in body weight, fat mass, or lean mass in patients with PHPT compared to matched controls. In addition, recent evidence from *in-vitro* studies suggests that higher levels of extracellular calcium inhibit adipogenesis [293]. An alternative explanation for our findings is that increased body weight predisposes to the development of PHPT. A positive association between parathyroid hormone concentrations and body weight has also been found in eucalcaemic populations [294-297]. In all cases 25OHD levels were inversely correlated with body weight, probably because vitamin D is fat soluble and is sequestered by adipose tissue. Thus increased body weight may promote vitamin D deficiency, resulting in secondary hyperparathyroidism [298]. Secondary hyperparathyroidism appears to increase the risk of developing parathyroid adenomata, a phenomenon that is well documented in the contexts of either renal failure [299] or long-term oral phosphate supplementation [300]. This hypothesis is supported by our earlier observation that increased body weight usually antedates the development of hypercalcaemia in PHPT [242].

There are limitations to our findings. Despite the high prevalence of PHPT, data from only 617 cases could be included in this meta-analysis. It is possible that selection bias may have influenced the results. Eight studies were carried out using patients referred for (or who subsequently had) parathyroid surgery. Three studies involved population screening. In two studies it was not clear how the patients were recruited. In the remaining studies, patients were attending endocrinology clinics. Subjects with more severe disease may therefore be

over-represented in this review. However, we believe, as discussed previously, that this would tend to minimise any increase in body weight because of the potential for anorexia associated with symptomatic disease. Ascertainment bias is also a possibility, if people who are overweight were more likely to have serum calcium measured, and as a consequence be found to have PHPT. We think this is an unlikely explanation for our findings given the consistency of the observation, and the fact that three of the studies were population-based screening studies.

In conclusion, this analysis of a substantial, existing body of published data confirms that increased body weight is consistently present in cohorts of subjects with PHPT, and suggests that the association is real. This observation raises important questions regarding the mechanism of the association, and provides a potential explanation for the increased prevalence of several cardiovascular risk factors and of increased cardiovascular mortality in patients with PHPT. Ultimately, these findings mandate a re-examination of the indications for parathyroidectomy in patients with asymptomatic disease, as although the updated NIH consensus conference guidelines [155] do not include these conditions as indications for parathyroidectomy, a number of editorialists do [239-241].



## **Chapter 7: Fat mass is an important predictor of parathyroid hormone levels in post-menopausal women**

### **Introduction:**

In Chapter 6, we showed that patients with PHPT were on average 3.1 kg heavier than age- and gender-comparable eucalcaemic controls in a meta-analysis of 17 studies that reported body weight or BMI in PHPT. The reason for this finding is not clear, and could theoretically operate in either direction: obesity could cause PHPT or vice versa. Previously, our group reported that in post-menopausal women with PHPT the majority of this weight difference (7.2 kg of 9.2 kg) was due to increased fat mass [158], and that higher body weight precedes the development of PHPT [242]. As discussed in Chapter 8, a growing body of evidence suggests that body weight and/or fat mass are inversely related to levels of serum 25OHD [211, 212, 297, 298, 301-306], which in turn are an important determinant of circulating levels of PTH [7, 210]. We therefore hypothesised that body weight/fat mass would be positively correlated with serum PTH levels in eucalcaemic subjects, and that this relationship would be mediated by vitamin D status.

Accordingly, we performed a cross-sectional analysis of data obtained in a cohort of healthy post-menopausal women, to determine the relationships among body weight, body composition, PTH, and vitamin D metabolites. We also measured regional body composition to determine whether visceral fat (trunk and pelvic fat) were more predictive than total body fat.

### **Methods:**

116 healthy post-menopausal women who were recruited for a study of the effects of hydrochlorothiazide on rates of BMD loss had a measurement of serum 25OHD. Full details of the study protocol have been published previously [307]. In brief, post-menopausal women who had not had a menstrual period in the last 5 years were recruited. Women who had undergone hysterectomy and were over the age of 55 with hormonally-confirmed post-menopausal status were also eligible. Exclusion criteria included disorders of calcium metabolism (including PHPT, Paget's disease, previously treated osteoporosis); renal, thyroid or hepatic dysfunction; other major systemic illnesses; the use of drugs known to affect calcium metabolism including the use of calcium supplements > 500 mg/day; and the use of

hormone replacement therapy in the previous 12 months. Participants were not excluded if they were vitamin D deficient or used vitamin D supplements.

Approximately 1200 women responded by telephone to initial advertisements and 301 completed screening questionnaires. 199 women were eligible to participate, but 14 elected not to proceed further. Thus, 185 women consented to enter the study. A random subset of 116 of these women had serum 25OHD measured, and data from this subgroup are included in the current analyses. The study was approved by the Auckland Ethics Committee.

Height was measured at baseline using a Harpenden stadiometer, and weight was recorded using electronic scales. Activity levels were recorded using a standardised questionnaire [308]. All women supplied a fasting blood sample and second-voided urine sample. Body composition and BMD of the lumbar spine, proximal femur and total body were measured with a DXA (Lunar DPX-L, Madison, WI, software version 1.3y). Serum 25OHD was measured by RIA (Incstar Corporation, Stillwater, MN). Serum 1,25(OH)<sub>2</sub>D was measured by RIA (Nichols Institute, San Juan Capistrano, CA) in a randomly selected subgroup of 54 women. Serum PTH was measured using an Allegro assay (Nichols Institute, San Juan Capistrano, CA). Fasting insulin levels were measured using an in-house RIA with an intraassay CV of 3.9%, and an interassay CV of 7.8%.

Insulin levels were logarithmically transformed because they were not normally distributed. Pearson correlation analysis was used to test for significant linear correlations between variables. Multivariate analysis was performed to determine the significant independent predictors of PTH, and 25OHD. Multivariate analysis was not performed for 1,25(OH)<sub>2</sub>D because of the small sample size ( $N=54$ ). Significance level was set at  $P < 0.05$ , all tests were two-tailed. Stepwise forward selection and backward elimination multiple regression analyses were performed to analyse the factors contributing most significantly to the variable of interest. Variables from Table 7.1 with  $P < 0.25$  in univariate analysis were included in the stepwise models with PTH, or 25OHD as the dependent variables. Variables with  $P < 0.05$  were retained in the model. Residuals were inspected. Models were chosen on the basis of biological plausibility and parsimony. Multiple linear regression was also used to determine the consequences of adjusting for vitamin D insufficiency and 25OHD levels. All statistical analyses were obtained using SPSS for Windows (SPSS Inc., Chicago, IL version 12.0.1) or the SAS software package (SAS Institute, Cary, NC version 9).

## **Results:**

Descriptive and biochemical characteristics of the study population are summarised in Table 7.1. The subgroup of 54 women who had 1,25(OH)<sub>2</sub>D measurements had similar baseline characteristics to the group as a whole (data not shown). We identified those variables that demonstrated significant correlations with PTH, 25OHD, and 1,25(OH)<sub>2</sub>D using Pearson correlation analysis. These correlations are presented in Table 7.2.

**Table 7.1: Descriptive and biochemical characteristics of the study group**

<i>N</i> = 116	Mean (SD)	Range
Age (years)	62.6 (5.9)	46-89
Height (cm)	162 (5.4)	150-177
Weight (kg)	67.4 (11.5)	44-110
Fat mass (kg)	27.3 (9.0)	8.0-56.1
Lean mass (kg)	36.7 (3.6)	29.9-46.2
Percent body fat (%)	41.7 (7.3)	18-59
Albumin-adjusted serum calcium (mmol/L)	2.27 (0.07)	2.11-2.47
Serum PTH (pmol/L)	2.7 (1.2)	1.0-7.6
Serum 25OHD (nmol/L)	54 (22)	12-115
25OHD < 25 nmol/L	7%	
25OHD < 50 nmol/L	47%	
Serum 1,25(OH) <sub>2</sub> D (pg/mL) <sup>a</sup>	36 (9)	10-60
Dietary calcium intake (mg/day)	1050 (540)	248-2948
Fasting serum insulin (mU/L)	7.7 (6.4)	0.8-51
Fasting serum glucose (mmol/L)	5.0 (1.1)	3.3-13.5
Serum albumin (g/L)	44 (2)	37-49
Alkaline phosphatase (U/L)	79 (18)	40-137
Total body BMC (kg)	2.2 (0.29)	1.47-2.95
<b>BMD (g/cm<sup>2</sup>)</b>		
L2-L4	1.06 (0.15)	0.66-1.63
Femoral neck	0.85 (0.12)	0.55-1.21
Total body	1.05 (0.08)	0.91-1.21

<sup>a</sup> Serum 1,25(OH)<sub>2</sub>D was measured in a random subset of 54 women.

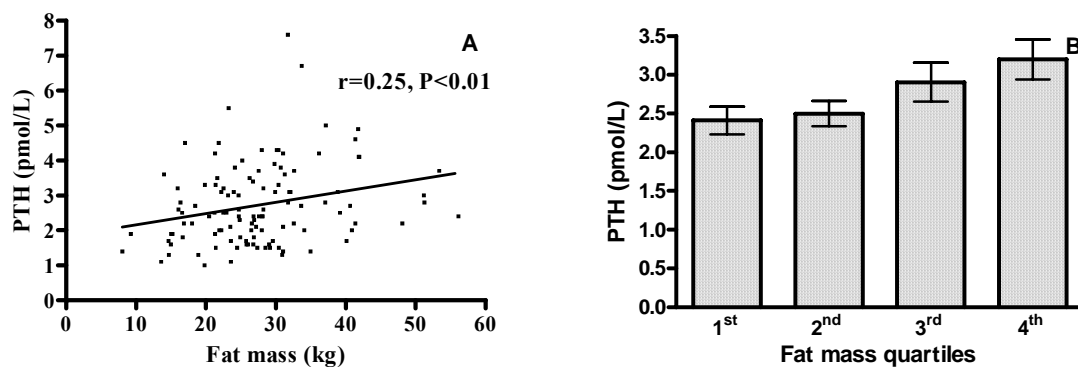
PTH was significantly positively correlated with body weight, regional and total fat mass, and percent body fat. The relationship between PTH and total fat mass is shown in Figure 7.1. There were significant negative correlations of PTH with activity levels, 25OHD, dietary calcium intake, and serum phosphate. For 25OHD, there were significant positive correlations with lumbar spine BMD and serum albumin, and significant negative correlations with PTH, total fat mass, trunk fat, and pelvic fat. For 1,25(OH)<sub>2</sub>D significant positive correlations were observed with 24-hour urine calcium and alkaline phosphatase, and a significant negative correlation with height. There were no significant correlations with PTH ( $r = 0.14$ ,  $P = 0.31$ ) or serum phosphate ( $r = -0.18$ ,  $P = 0.20$ ).

**Table 7.2: Pearson's correlations between vitamin D metabolites, PTH, and other variables**

	PTH	25OHD	1,25(OH) <sub>2</sub> D
Age	-0.04	-0.03	-0.19
Weight	0.20*	-0.14	-0.03
Height	-0.02	0.02	-0.33*
Activity level	-0.19*	0.02	-0.14
Serum 25OHD	-0.22*		-0.16
Serum 1,25(OH) <sub>2</sub> D	0.14	-0.16	
Albumin-adjusted serum calcium	-0.02	0.01	0.21
Serum phosphate	-0.28**	0.12	-0.18
Serum PTH		-0.22*	0.14
Serum albumin	-0.06	0.28**	-0.02
Serum ALP	0.06	-0.16	0.34*
24 hour urine calcium	-0.04	0.12	0.36*
Dietary calcium	-0.26**	0.03	-0.25
Fasting serum glucose	0.03	0.05	0.11
Serum insulin	-0.04	-0.06	0.19
Pelvis fat	0.25**	-0.23*	-0.01
Trunk fat	0.25**	-0.20*	0.03
Total fat mass	0.25**	-0.18*	-0.02
Percent body fat	0.30**	-0.16	-0.03
Lean mass	-0.04	-0.02	-0.07
L2- L4 BMD	-0.05	0.19*	-0.16
Femoral neck BMD	-0.02	0.04	-0.08
Total body BMD	0.07	0.02	-0.10
Total body BMC	0.06	0.06	-0.24

\* P < 0.05, \*\* P < 0.01. For PTH and 25OHD N=116; for 1,25(OH)<sub>2</sub>D N=54.

**Figure 7.1: Relationship between PTH and fat mass**



Panel A shows a scatter plot of PTH versus fat mass with line of best fit and Panel B the mean PTH by quartile of fat mass. The error bars represent the SEM.

In order to determine whether the relationship between PTH and fat mass is dependent on the presence of vitamin D insufficiency (25OHD < 50 nmol/L as recommended by a recent WHO report [309]), we created a model with PTH as the dependent variable, and fat mass and a coded variable representing the presence or absence of vitamin D insufficiency as the explanatory variables. Using this model there was no change in the relationship between PTH and fat mass after adjusting for the presence of vitamin D insufficiency (for model of PTH and fat mass  $r^2 = 0.06$ ,  $P = 0.007$ ; for model of PTH, fat mass, and vitamin D insufficiency  $r^2 = 0.07$ , fat mass  $P = 0.015$ , vitamin D insufficiency  $P = 0.36$ ). We used a similar approach to determine whether the relationship between PTH and fat mass is dependent on 25OHD levels expressed as a continuous variable. We created a model with PTH as the dependent variable, and fat mass and 25OHD as the explanatory variables, and found that there was no change in the relationship between PTH and fat mass after adjusting for 25OHD levels (for model of PTH, fat mass, and 25OHD  $r^2 = 0.09$ , fat mass  $P = 0.019$ ; 25OHD  $P = 0.055$ )

The results of the multivariate analyses are presented in Table 7.3. For PTH, only percent body fat, serum phosphate, and dietary calcium intake remained significantly related to PTH on multivariate analysis. 25OHD did not meet the criteria to be retained in the model although statistical significance was approached ( $P = 0.08$ ). This model accounted for 17% of the variation in PTH. When we adjusted for 25OHD levels, by entering 25OHD into the model, there was no change in the relationship between PTH and percent body fat. For 25OHD, on multivariate analysis serum albumin and pelvic fat mass remained significantly related to 25OHD. PTH did not meet the criteria to be retained in the model although statistical significance was approached ( $P = 0.06$ ). This model explained 13% of the variance in 25OHD. Lumbar spine BMD was not entered into the multivariate model as it is unlikely to influence 25OHD levels. When we adjusted for PTH levels, by entering PTH into the model, there was little change in the relationship between 25OHD and pelvic fat mass.

**Table 7.3: Predictors of PTH and 25OHD in multivariate regression models**

Independent Variable	Regression Coefficient	95% CI	P	Partial r <sup>2</sup>
Model with <b>PTH</b> as dependent variable, P for model < 0.001, r <sup>2</sup> for model 0.17				
Percent body fat	0.04	0.01 to 0.06	0.020	0.10
Serum phosphate	-1.87	-3.51 to -0.22	0.026	0.04
Dietary calcium	-0.0004	-0.0008 to -0.0000	0.041	0.03
Model with <b>25OHD</b> as dependent variable, P for model < 0.001, r <sup>2</sup> for model 0.13				
Serum albumin	2.23	0.59 to 3.87	0.008	0.07
Pelvic fat mass	-3.13	-5.61 to -0.66	0.014	0.05

### **Discussion:**

We found that PTH levels are positively correlated with body weight and fat mass in a population of healthy post-menopausal women. Fat mass appears to be the major contributor to this relationship because it is a stronger predictor of PTH than is body weight, and there was no relationship between PTH levels and lean mass. These results extend our previous finding (described in Chapter 6) of increased body weight in PHPT, a state of PTH excess, by demonstrating a positive association between body weight and PTH in post-menopausal women with normal parathyroid function. This relationship between PTH and body weight/fat mass has also been seen in several recent studies in eucalcaemic subjects [212, 294-297, 305, 310] including random samples from the general population [294, 295], elderly nursing home residents [296], healthy adult volunteers [297], population-based studies [212, 310] and post-menopausal women attending osteoporosis clinics [305].

The explanation for the positive relationship between PTH and fat mass is not clear. One possibility is that elevated PTH levels arise as a consequence of increased body weight as described in Chapter 6. Our previous report that women with PHPT were heavier throughout their adult lives than age-matched controls [242] suggests that increased body weight promotes PTH excess and that PHPT may be a long-term complication of increased body weight. A possible mechanism by which this relationship may be mediated is via altered vitamin D metabolism [298]. In Chapter 8, we report an inverse relationship between fat mass and serum 25OHD. Other researchers have previously reported, in a variety of study populations, similar inverse relationships between body weight, BMI and/or fat mass and serum 25OHD [211, 212, 297, 298, 301-306]. This relationship may occur because 25OHD is

fat soluble, leading to increased sequestration in adipose tissue in obese individuals [301, 303]. Other possible explanations would be that overweight people have decreased exposure to sunlight because of their choice of clothing, or because of decreased exercise levels and mobility in heavier individuals. Low 25OHD levels promote secondary hyperparathyroidism [7] which may in turn increase hepatic catabolism of 25OHD, thereby further lowering 25OHD levels [311]. Thus, increased body weight may result in secondary hyperparathyroidism, which may in turn increase the risk of developing parathyroid adenomata, a phenomenon well documented in renal failure [299] and long-term oral phosphate supplementation [300]. The inverse relationship between body weight/fat mass and 25OHD therefore provides a potential explanation for the findings that PTH levels are related to body weight, and patients with PHPT are heavier than their eucalcaemic peers.

In the current study, levels of 25OHD were negatively correlated with body weight and measurements of total and regional body fat. The correlations with pelvic, trunk and total fat mass were stronger than those with body weight and lean mass, suggesting that fat mass is the main contributor to this relationship. These results are similar to those from a larger study, described in Chapter 8, of more than 1600 post-menopausal women with similar baseline clinical characteristics, in whom we report that 25OHD had significant negative correlations with measures of fat mass and body weight, but no relationship with lean mass. In addition, in the current study, adjustment for the presence of vitamin D insufficiency, or 25OHD levels, did not substantially change the relationships between PTH and measures of fat mass. This has not been a consistent observation in previous studies. BMI [294] and body weight [305] and fat mass [212] were independent predictors of PTH after controlling for 25OHD levels in three studies, whereas in a group of elderly institutionalised patients the PTH/body weight relationship was dependent on hypovitaminosis D [296]. These results suggest that hypovitaminosis D may not be the only mediator of the relationship between PTH and fat mass.

An alternative explanation for the association between PTH and body fat is that elevated PTH levels cause increased fat mass. As described in Chapter 6, increased PTH levels may cause increased intracellular calcium levels in adipocytes, thereby inhibiting lipolysis and promoting insulin resistance [290, 310]. However, recent evidence from *in vitro* studies suggests that higher levels of extracellular calcium inhibit adipogenesis [293]. Further, if this hypothesis was correct, elevated PTH levels of any cause would promote increased fat mass

and body weight, which may be corrected by normalising the PTH levels (replenishing vitamin D stores in vitamin D insufficiency with secondary hyperparathyroidism, or parathyroidectomy in PHPT). We are not aware of any data that demonstrate reduction in body weight after correction of PTH excess. In one study that reported on changes in body composition three years after parathyroidectomy, there was no change in body weight, fat mass, or lean mass in patients with PHPT compared to matched controls [164]. Another possible explanation for the association between PTH and body fat is that increased fat mass in post-menopausal women is associated with higher oestrogen levels, via aromatase activity in adipocytes. However, the available evidence suggests that oestrogen does not influence PTH production in the long term [312, 313], and a positive association between body weight and PTH has been reported in populations that include pre-menopausal women, in whom aromatase-derived oestrogen contributes insignificantly to total circulating oestrogen levels, and men. Thus, levels of endogenous oestrogen are unlikely to mediate the PTH/fat mass relationship that we observed.

In contrast to the relationships between fat mass, 25OHD, and PTH there was no relationship between 1,25(OH)<sub>2</sub>D and any of these variables. In fact 1,25(OH)<sub>2</sub>D was only significantly correlated with ALP, height, and 24 hour urine calcium. It is surprising that 1,25(OH)<sub>2</sub>D levels are not correlated with serum phosphate or PTH, the factors which tightly regulate the activity of the 1 $\alpha$ -hydroxylase enzyme responsible for synthesis of 1,25(OH)<sub>2</sub>D, especially since 1,25(OH)<sub>2</sub>D and serum phosphate themselves are important regulators of *PTH* gene expression [314]. A possible explanation is that levels of free 1,25(OH)<sub>2</sub>D, which we did not measure, may show different correlations than the levels of total hormone, which comprises protein-bound and free components [315, 316]. Other researchers reported from larger studies that 1,25(OH)<sub>2</sub>D had significant correlations with PTH, serum phosphate, and 25OHD but not body weight [305], and significant correlations with BMI and fat mass [297]. The discrepancy between our findings and the findings of these other studies may be in part due to the size of our study.

There are limitations to our findings. We have performed multiple statistical tests, and there is a possibility that some of the correlations which are statistically significant have arisen due to chance. Nevertheless, due to the strength of the important correlations discussed above, their consistency with previous studies, and their biological plausibility, we believe they are real. The study is moderately sized with 80% power (at the 5% significance level) to detect



univariate correlations of at least 0.26, and so there is the possibility that some correlations will have been misclassified as not statistically significant due to a Type II error. The possibility of selection bias also arises. Participants in this study may have chosen to take part because they were more aware of health issues and healthier than average, and therefore have higher vitamin D levels and BMD. Conversely, participants may have chosen to take part because they believed they were at higher risk of osteoporosis because of family history or background of osteoporosis or poor health, and therefore had lower BMD or vitamin D levels. However the mean BMD and 25OHD level were similar to those we found in a much larger study of women recruited from the same general population, described in Chapter 8.

In summary, body weight, and more specifically fat mass, is an important determinant of levels of PTH (positive relationship) and 25OHD (inverse relationship) in healthy postmenopausal women. In contrast 1,25(OH)<sub>2</sub>D was not related to these variables. The relationship between PTH and fat mass appears to be independent of hypovitaminosis D, and of the inverse association between fat mass and 25OHD. The positive association between PTH and fat mass in eucalcaemic post-menopausal women is consistent with the observation we recently made that body weight is increased in PHPT. Taken together with our previous observation that increased body weight precedes the development of PHPT, these findings suggest that increased body weight and obesity may predispose to the development of PHPT.

## **Chapter 8: Determinants of vitamin D status in middle-aged and older men and in older women**

In the previous two Chapters, I described work that explored the determinants of serum PTH levels and the relationship between PTH and body weight. In this Chapter, I turn to the determinants of 25OHD and the relationship between 25OHD and body weight.

### **Introduction:**

As discussed in Chapter 5, vitamin D insufficiency is common in older people and is considered to contribute to bone loss and muscle weakness, thus leading to fractures. Studies performed in the Northern Hemisphere or in regions distant from the equator have suggested that there are significant seasonal variations in 25OHD levels, with corresponding seasonal fluctuations in PTH levels, markers of bone turnover and BMD [187, 188, 317]. Since sunlight exposure is the principal source of vitamin D, and this is affected by geographical location and lifestyle, studies in Northern Europe and the United States are not likely to predict the prevalence of vitamin D deficiency in a subtropical climate, such as northern New Zealand. Indeed, few studies have explored circannual variation or the determinants of 25OHD in subtropical climates, and, while many studies have reported 25OHD levels in cohorts of post-menopausal women, there are comparatively few studies reporting data from middle-aged or older men. Furthermore, some reports have suggested that there may be gender differences in 25OHD levels and in the relationships between 25OHD, calcitropic hormones, and indices of bone metabolism [211, 302, 318-320].

We have measured 25OHD levels at recruitment into studies of calcium supplementation in normal post-menopausal women and middle-aged and older men living in Auckland, New Zealand (latitude 37°S). We set out to determine the “normal” levels of 25OHD in these cohorts of independent-living, New Zealand men and women, and the effects of season, body composition, and other factors on these levels. We then compared the data from the cohorts of men and women to determine whether there are gender differences in 25OHD levels, or the relationships between 25OHD, body composition and metabolic indices.

## **Methods:**

### ***Participants:***

Between October 1997 and June 2000, 1606 healthy, post-menopausal women living independently in Auckland, underwent biochemical assessment for a study of calcium supplementation. Participants were women born between 1915 and 1929 whose names were randomly selected from the New Zealand electoral roll. Newspaper advertisements and presentations to women's groups were also used for recruitment. Women were eligible for inclusion in the study if they were greater than five years post-menopausal, aged at least 55 years, and their life expectancy was greater than five years. Between January 2004 and May 2005, 378 healthy, independent-living, middle-aged or older men underwent biochemical assessment for a study of calcium supplementation. Participants were volunteers aged over 40 years who responded to newspaper advertisements. In both studies, exclusion criteria included significant hepatic, renal or thyroid dysfunction, concurrent major systemic illness including malignancy, undiagnosed diabetes mellitus, or metabolic bone disease. Further exclusion criteria included the use of medication known to interfere with calcium metabolism, the use of cholecalciferol supplements in doses exceeding 1000 IU/day, the current use of glucocorticoids, or the use of testosterone, hormone replacement therapy, anabolic steroids, fluoride, or bisphosphonates in the previous one year. Additionally, men were excluded from participation if they had coronary heart disease, an estimated five year cardiovascular risk of greater than 15% [321], or were receiving therapy for hyperlipidaemia. The studies received ethical approval from the Auckland Ethics Committee.

3935 women initially responded to the invitation letter and were sent a questionnaire. From this group, 1606 women (41%) were enrolled in this study. The reasons for non-inclusion were failure to return completed questionnaire (n=1514); exclusion on the basis of clinical or laboratory selection criteria (n=513); unwilling or unable to have blood samples performed (n=186), and patient decision not to participate (n=116). 909 men initially responded to the advertisements and were sent a screening questionnaire. From this group, 378 (42%) were enrolled in the study. The reasons for non-inclusion were failure to return a completed questionnaire (n=339); exclusion on the basis of clinical or laboratory selection criteria (n=111); unwilling or unable to have blood samples performed (n=31), and patient decision not to participate (n=50).

## **Measurements:**

Lifestyle information was collected at a pre-study visit, including a physical activity record [308]. Height was measured at baseline using a Harpenden stadiometer, weight was recorded using electronic scales, and all participants supplied a fasting blood sample. Body composition was measured using a Lunar Expert DXA, software version 1.7 (GE Lunar, Madison, WI) in the women and using a Lunar Prodigy DXA (GE Lunar, Madison, WI) in the men. Measurements of the monthly, averaged, erythemally-weighted UV dose measured near Auckland at latitude 36.5°S were supplied by Dr. Richard McKenzie of the National Institute of Water and Atmospheric Research. Serum 25OHD was measured by RIA (DiaSorin, Stillwater, MN) in the women and in the first 252 men, then using a chemiluminescent assay (Nichols, San Juan Capistrano, CA) in the last 126 men.

All 25OHD samples from both studies were measured in one laboratory which takes part in, and meets the performance targets for, the Vitamin D External Quality Assessment Scheme (DEQAS) [322]. The interassay CV for the DiaSorin assay was 8.8% and for the Nichols assay was 7.5% in our laboratory. Because there was a change in assay during the studies, we developed equations to allow the interconversion of results obtained from the two assays. 25OHD had previously been measured using both assays in 215 samples in our laboratory. The range of results was 0-174 nmol/L using the Nichols assay and 0-141 nmol/L using the DiaSorin assay. The results were compared using type II linear regression. The results from both assays were highly correlated (Spearman correlation coefficient 0.89,  $P < 0.001$ ). All data obtained using the Nichols assay were converted to predicted DiaSorin results using the derived equation  $\text{DiaSorin} = \text{Nichols} * 0.75 + 5.6$ .

## **Statistics:**

ANOVA was used to test for monthly and seasonal differences in variables. Significant main effects were further investigated using the method of Tukey. The temporal relationship between 25OHD and UV levels was investigated using standard autoregressive techniques. Pearson correlation analysis was used to test for significant associations between 25OHD and the other variables measured. The chi-square procedure was used to compare the frequency of low 25OHD across month and season. Significance level was set at  $P < 0.05$  and all tests were two-tailed. A stepwise regression analysis was performed to analyse the factors contributing most significantly to 25OHD. Factors with  $P < 0.25$  in univariate analysis were included in a

stepwise model with 25OHD as the dependent variable. ANCOVA and multiple linear regression were used to test for differences in 25OHD levels between genders. All statistical analyses were obtained using the SAS software package (SAS Institute, Cary, NC version 9.1).

## **Results:**

### ***Women:***

Descriptive and biochemical characteristics of the study population are summarised in Table 8.1. The majority of this group were of European descent (1603 women), with two women being of Asian and one of Polynesian ethnicity. Of note, the mean serum 25OHD level was 51 nmol/L, equivalent to the WHO criterion for vitamin D sufficiency [309]. The mean serum 25OHD level decreased with age, as shown in Table 8.2.

**Table 8.1: Descriptive and biochemical characteristics of the study populations**

	Men (N=378)		Women (N=1606)	
	Mean (SD)	Range	Mean (SD)	Range
Age (years)	57 (11)	40-88	74 (4)	54-86
Height (cm)	176 (7)	158-194	159 (6)	138-183
Weight (kg)	82 (13)	54-136	67 (11)	40-120
BMI (kg/m <sup>2</sup> )	26.4 (3.4)	17.8-41.2	26.4 (4.3)	15.9-46.9
Lean mass (kg)	59 (7)	42-81	36 (4)	24-51
Fat mass (kg)	19 (8)	3-48	27 (10)	3-74
Percent fat	24 (7)	6-42	41 (8)	8-64
Dietary calcium (mg/d)	880 (473)	106-2631	855 (384)	71-3186
Physical activity (METS)	32 (5)	26-57	34 (4)	23-60
Gardening (hours/week)	2.0 (3.1)	0-25	4.2 (5.1)	0-50
Other leisure activities (hours/week)	5.7 (5.3)	0-43	5.3 (5.6)	0-56
Serum creatinine (mmol/L)	0.09 (0.01)	0.06-0.15	0.09 (0.01)	0.05-0.17
Glucose (mmol/L)	5.0 (0.5)	3.9-6.9	5.1 (0.7)	2.8-10.1
Adjusted serum calcium (mmol/L)	2.33 (0.08)	2.08-2.53	2.28 (0.09)	2.01-2.60
Albumin (g/L)	44 (3)	35-51	42 (2.2)	35-50
25OHD (nmol/L)	85 (31)	17-200	51 (19)	10-145

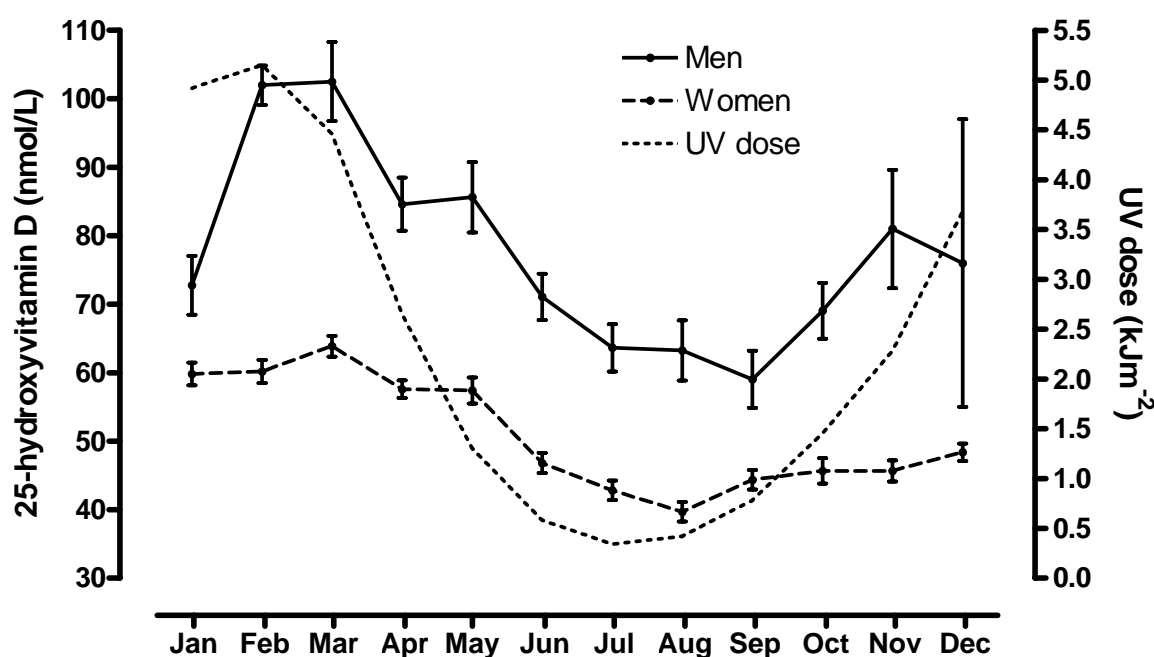
**Table 8.2: Mean levels of 25OHD by age range**

Age	Men			Age	Women		
	Mean (SD) <sup>a</sup>	Range	N		Mean (SD) <sup>a</sup>	Range	N
40-50	84.9 (34.8)	17 to 198	116				
50-60	87.1 (29.9)	30 to 172	127				
60-70	84.2 (27.7)	38 to 200	79	60-70	54.3 (20.5)	10 to 128	398
70+	83.7 (29.5)	38 to 180	56	70-80	50.9 (18.7)	10 to 145	1083
				80+	44.1 (19.8)	15 to 135	125

<sup>a</sup>25OHD units are nmol/L

Figure 8.1 shows the monthly variation in UV radiation and 25OHD. There are substantial circannual variations in UV dose with the highest levels in summer, as expected. The peak level in February was 15 times the nadir level in July. The monthly mean 25OHD levels closely parallel this seasonal fluctuation, with a lag of 1-2 months [modelled lag 1.7 months (95% CI 1.1 to 2.3)]. The percentages of women with vitamin D deficiency (25OHD < 25 nmol/L) and vitamin D insufficiency (25OHD < 50 nmol/L) in each month of sampling are shown in Table 8.3. In summer 0-3% of women were vitamin D deficient and 28-58% were vitamin D insufficient, while in winter these figures increased to 6-16% and 56-74% respectively (P < 0.0001).

**Figure 8.1: Monthly variation of UV dose and 25OHD levels**



UV dose is shown as the dotted line, 25OHD levels in men as the solid line, and 25OHD levels in women as the dashed line. Data are mean 25OHD levels for each month with error bars representing the SEM.

**Table 8.3: Frequency of low 25OHD levels by month of sampling**

Month	Men			Women		
	N	Vitamin D Deficient (%)	Vitamin D Insufficient (%)	N	Vitamin D Deficient (%)	Vitamin D Insufficient (%)
January	30	0.0	16.7	123	0.0	30.1
February	134	0.0	3.7	127	1.6	28.3
March	18	0.0	0.0	155	0.6	22.6
April	47	0.0	4.3	172	1.2	31.4
May	26	3.8	3.8	115	0.0	36.5
June	45	2.2	20.0	123	5.7	56.1
July	19	0.0	10.5	123	13.0	68.3
August	4	0.0	0.0	140	16.4	74.3
September	23	0.0	26.1	134	9.7	61.2
October	21	0.0	14.3	110	12.7	58.2
November	9	0.0	11.1	152	6.6	64.5
December	2	0.0	0.0	132	3.0	57.6

Vitamin D deficiency is 25OHD < 25 nmol/L. Vitamin D insufficiency is 25OHD < 50 nmol/L

We identified those variables from Table 8.1 that demonstrated significant correlations with 25OHD using Pearson correlation analysis (Table 8.4). There were significant negative correlations between 25OHD and subject age, weight, BMI, fat mass, and percentage body fat. There were significant positive correlations between 25OHD and physical activity and time spent gardening. Biochemical variables that exhibited significant correlation with 25OHD were ALP and fasting serum glucose. There was no significant correlation between 25OHD and lean mass. On multivariate analysis age, fat mass, physical activity, and month of blood sample remained significantly related to 25OHD (Table 8.5). This four variable model accounted for 21% of the variation in 25OHD. Excluding season from the model did not change the relationships between age, fat mass, physical activity and 25OHD.

### **Men:**

Descriptive and biochemical characteristics of the study population are shown in Table 8.1. 346 (92%) men were of European descent, 12 (3%) were of East Asian descent, 12 (3%) were of Maori/Polynesian descent, and 8 (2%) were of Indian descent. The mean serum 25OHD level (SD) was 85 (31) nmol/L, consistent with vitamin D sufficiency. The mean 25OHD level (SD) was higher in Europeans [87 (30) nmol/L] than non-Europeans [66 (31) nmol/L],  $P < 0.001$ . The mean 25OHD level did not decrease with age, as shown in Table 8.2.

Figure 8.1 shows the monthly variation of UV and 25OHD levels. The mean 25OHD levels closely parallel the seasonal fluctuation in UV levels, with a lag of 1-2 months. Vitamin D

**Table 8.4: Pearson's correlations between 25OHD and other variables**

	Men	Women
Age	-0.03	-0.15**
Height	0.11	0.04
Weight	-0.07	-0.11**
BMI	-0.14*	-0.14**
Lean mass	0.09	0.01
Fat mass	-0.21**	-0.15**
Percent fat	-0.23**	-0.15**
Dietary calcium	0.00	0.00
Physical activity	0.20**	0.09**
Gardening hours	0.14*	0.08**
Serum creatinine	0.07	0.02
Glucose	-0.06	-0.05*
Adjusted serum calcium	-0.13*	0.00
ALP	-0.12*	-0.10**
Serum phosphate	0.03	-0.04
Albumin	0.08	0.04

\* P< 0.05, \*\* P< 0.01.

**Table 8.5: Predictors of 25OHD levels in multivariate regression models**

Independent Variable	Regression Coefficient	95% CI	P	Partial r <sup>2</sup>
<b>Model for women with 25OHD as dependent variable, for model P&lt; 0.0001, r<sup>2</sup>= 0.21</b>				
Month of blood sample			<0.0001	0.16
Age (years)	-0.72	-0.92 to -0.51	<0.0001	0.02
Physical activity (METS)	0.28	0.08 to 0.48	0.0056	0.01
Fat mass (kg)	-0.32	-0.42 to -0.23	<0.0001	0.03
<b>Model for men with 25OHD as dependent variable, for model P&lt; 0.0001, r<sup>2</sup>= 0.33</b>				
Month of blood sample			<0.0001	0.25
Percent fat	-1.06	-1.52 to -0.60	<0.0001	0.05
Serum albumin	2.09	0.87 to 3.31	0.0009	0.02
Physical activity (METS)	0.92	0.31 to 1.53	0.0036	0.01
<b>Model for combined datasets of men and women with 25OHD as dependent variable, for model P&lt; 0.0001, r<sup>2</sup>= 0.42</b>				
Gender (0=male 1=female)	-13.3	-17.7 to -8.9	<0.0001	0.26
Month of blood sample			<0.0001	0.11
Percent fat	-0.43	-0.55 to -0.32	<0.0001	0.02
Age	-0.51	-0.67 to -0.35	<0.0001	0.02
Physical activity (METS)	0.39	0.19 to 0.58	0.0001	0.01
Serum albumin	0.53	0.13 to 0.94	0.01	0.002

insufficiency and deficiency were uncommon in the male cohort (Table 8.3). Only two men (0.5%) were vitamin D deficient, and 34 (9%) were vitamin D insufficient. In summer, the prevalence of vitamin D insufficiency ranged from 0-17% and no men were vitamin D



deficient, while in winter the prevalences ranged from 0-20% and 0-2%, respectively. 0/2 men who were vitamin D deficient and 24/34 men who were vitamin D insufficient were of European descent.

We identified variables from Table 8.1 that showed significant correlations with 25OHD using Pearson correlation analysis. There were significant negative correlations between 25OHD and percentage body fat, fat mass, and BMI and significant positive correlations between 25OHD and physical activity and time spent gardening. There were no significant correlations between 25OHD and age, body weight, or lean mass. Biochemical variables that exhibited significant correlation with 25OHD were ALP and adjusted serum calcium. On multivariate analysis, the month of the blood sample, percentage body fat, serum albumin, and physical activity were significantly related to 25OHD. This four variable model accounted for 33% of the variation in 25OHD levels.

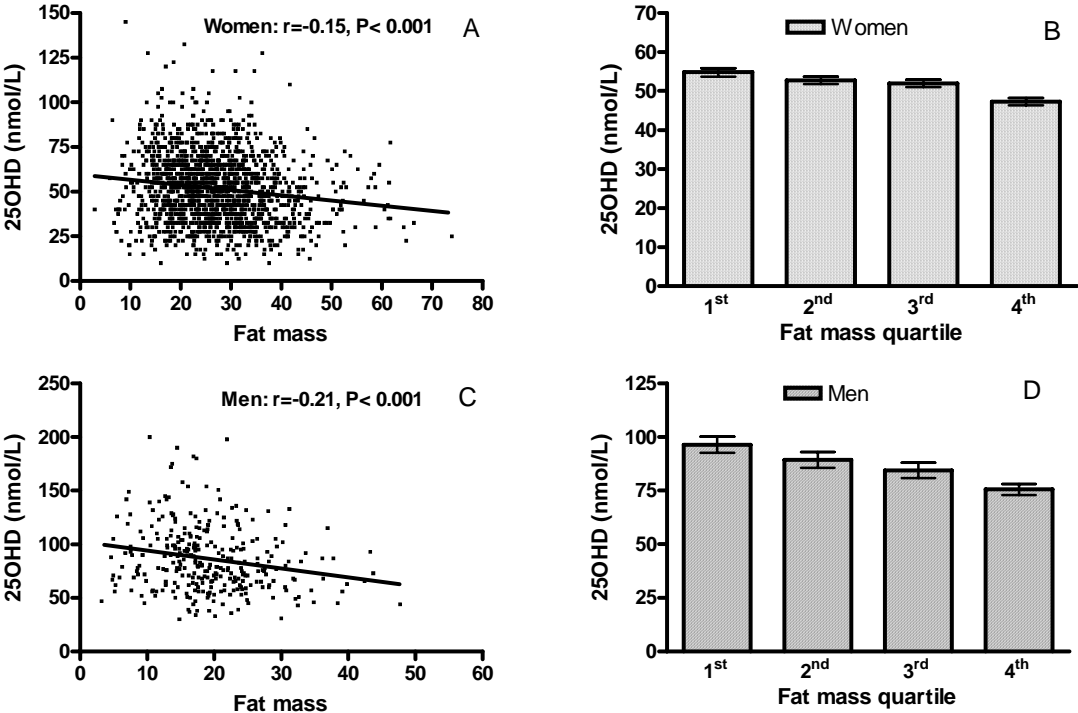
### ***Comparisons between the women and men:***

We compared the data from the cohorts of women and men. Although the majority of participants in both cohorts were of European descent, a higher proportion of women were of European descent ( $P < 0.001$ ). Table 8.1 shows that the women were older, shorter, and weighed less than the men, with the expected differences in body composition, such that the women had higher fat mass and greater percentage body fat than the men. However the mean BMI, levels of physical activity, dietary calcium and serum biochemistry of the cohorts were similar. Figure 8.1 shows that there is similar monthly variation of 25OHD levels in women and men, but that, at each monthly time point, 25OHD levels are higher in men than in women. Since the differences in age and month of blood sampling between the cohorts may potentially have confounded these results, we adjusted for these differences by ANCOVA and found that the differences in 25OHD levels between the men and women remained significant ( $P < 0.001$ ).

Table 8.4 shows that correlations of similar strength and direction were observed between 25OHD and percentage body fat, fat mass, BMI, and physical activity in both cohorts. Lean mass was not correlated with 25OHD in either cohort. Differences between the cohorts occurred in the correlations between 25OHD and age, and 25OHD and weight. There was a significant negative correlation between 25OHD and age in the women but not in the men. The inverse correlations between 25OHD and weight were of similar strength in both cohorts

but the relationship was only significant in the much larger cohort of women. The major determinants of 25OHD levels in both men and women on multivariate analysis were the month of blood sampling, measures of fat mass, and physical activity. The relationship between 25OHD and fat mass is shown in Figure 8.2.

**Figure 8.2: The relationship between 25OHD and fat mass**



Panel A shows a scatter plot of 25OHD versus fat mass with line of best fit in the women and Panel B the mean 25OHD by quartile of fat mass in the women. Panel C shows a scatter plot of 25OHD versus fat mass with line of best fit in the men and Panel D the mean 25OHD by quartile of fat mass in the men. The error bars represent the SEM.

Finally, we examined determinants of 25OHD levels in the combined datasets. Factors that were significantly related to 25OHD in each of the two cohorts were entered into the multivariate analysis of the combined datasets (Table 8.5). Gender, month of blood sampling, percentage body fat, age, physical activity, and serum albumin all were significantly related to 25OHD. This six variable model accounted for 42% of the variation in 25OHD levels. After adjusting for the differences between the cohorts in month of blood sampling, percentage body fat, age, physical activity, and serum albumin, the mean (95% CI) 25OHD level remained significantly higher in men [67 (63 to 71) nmol/L] than in women [54 (53 to 55) nmol/L]. Thus, gender remained an independent predictor of 25OHD levels after adjusting for potential confounders. We repeated the ANCOVA and multiple regression analyses

restricting the analyses to men and women of similar age range (60-80 years) using the same selection criteria and obtained similar results. The unadjusted mean (SD) 25OHD level for 131 men was 84 (29) nmol/L and for 1364 women was 52 (19) nmol/L. After adjusting for differences in the month of blood sampling, differences in percentage fat, exercise levels, and age between the cohorts, the difference in 25OHD levels between genders remained.

## **Discussion:**

This study shows that up to 74% of healthy, independent, post-menopausal, Auckland women have vitamin D insufficiency during winter and 30% during summer. However, in independent-living, middle-aged and older, Auckland men vitamin D deficiency was rare, and the prevalence of vitamin D insufficiency in winter was low. There were significant seasonal variations in 25OHD levels in both cohorts, but men had significantly higher levels of 25OHD than women after adjusting for potential confounding factors including differences in age, body composition, physical activity and the month of blood sampling. In both men and women, the major determinants of 25OHD levels were the month of blood sampling, measures of fat mass, and levels of physical activity. In women but not men, age was also an independent predictor of 25OHD levels— in women, 25OHD levels fell with age whereas, in men, there was no decline in 25OHD levels with age.

The findings of high rates of vitamin D deficiency in the cohort of women confirms the findings of a number of smaller studies performed in Australia [323, 324] and New Zealand [325], and a recent New Zealand population-based study [306] which show that, despite substantial amounts of sunshine throughout the year, a significant proportion of the independent-living elderly population have vitamin D insufficiency. Geographical location within New Zealand may also play a role in vitamin D status. The North Island of New Zealand receives approximately 10% more UV radiation than the South Island in summer, and twice the amount in winter [326], and thus it is likely that the percentage of women with suboptimal vitamin D levels increases substantially with progression south. In a study performed in 38 women living in Dunedin, New Zealand (latitude 46<sup>0</sup>S), 26% women in summer and 69% in winter had 25OHD levels less than 40 nmol/L [325]. In our study the corresponding values were 17% in summer and 43% in winter. In a recently published population-based study from New Zealand, the mean 25OHD level in 266 women over 65 years of age was 43 nmol/L and 58% had 25OHD levels less than 50 nmol/L [306].

There are few comparable studies of 25OHD levels in healthy, middle-aged and older men. Scragg et al. in 1992 reported that in a randomly selected sample of 295 New Zealand men aged 35-64 years the mean 25OHD level was 39.8 nmol/L [327]. Rockell et al. recently reported that a population-based sample of 419 men aged 45-64 years and 206 men aged over 65 years had mean 25OHD levels of 52 nmol/L and 55 nmol/L respectively [306]. There are no other studies of similar populations of men from Australasia that we are aware of. Table 8.6 summarises results of studies of independent-living, middle-aged or older men from the United States of America (USA) and Europe, all of which were carried out in sites at higher latitude than Auckland. Our results show similar 25OHD levels to the USA studies, with a lower rate of vitamin D insufficiency than was observed in the European studies. The comparable (low) rates of vitamin D insufficiency in our study and those from the USA, despite lower UV exposure in the USA centres, may reflect the fortification of food with vitamin D in the USA.

**Table 8.6: Studies from Europe and USA that report 25OHD levels in cohorts of men**

Reference	Location	Latitude (° north)	N	Age (years) (mean or range)	Mean 25OHD (nmol/L)
[318]	Europe	35-61	414	71-76	39 <sup>a</sup>
[210]	France	43-51	765	52	62 <sup>b</sup>
[328]	Southern France	47	881	58	70
[320]	Northern Italy	44	497	20-85+	60
[187]	Germany	49	276	65	52
[212]	Amsterdam	52	237	75	57
[329]	Baltimore, USA	39	167	57	89
[211]	Boston, USA	42	182	71	82
[302]	Framingham, USA	42	290	74	82

<sup>a</sup> 25OHD measured between January and March

<sup>b</sup> 25OHD measured between November and April

The major determinants of 25OHD levels in men were the month of blood sampling, levels of physical activity, and adiposity (percentage body fat). In women, the major determinants of 25OHD levels were also the month of blood sampling, levels of physical activity, and adiposity (total fat mass) but, in addition, age was an independent predictor of 25OHD levels. Seasonal fluctuation in serum 25OHD is well described in both the young and elderly, and is associated with both personal sun exposure and UV levels [188, 330]. In humans, the major source of vitamin D is from UV-B-induced photolysis of steroid precursors in the skin. Seasonal variation in UV-B levels occurs because the lower angle of incidence of incoming solar radiation during winter results in UV rays travelling a greater distance through the

atmosphere, which in turn increases atmospheric absorption of UV radiation. Seasonal changes in cloud cover (with greater cloud cover in Auckland in winter) may also contribute to the increased atmospheric absorption of UV radiation. In addition, exposure of the skin to UV-B is generally decreased during the colder winter months because more clothes are worn. The formation of vitamin D in the skin in response to UV-B radiation is affected by an individual's age, degree of skin pigmentation, and the intensity of sun exposure [7]. There is also a small dietary contribution to 25OHD levels. The Australia New Zealand Food Authority has estimated the mean population vitamin D intake is only 80-96 IU/day [331] which is comparable to other populations with similar diets and unlikely to significantly impact on 25OHD levels [332]. We have clearly demonstrated seasonal variation in 25OHD levels in both men and women, with a "lag" of 6-8 weeks between the maximum monthly UV dose and the peak 25OHD level. This lag period has been described previously [189, 333]. The time taken to establish a steady state of a compound is approximately 3 to 4 times its half-life, which for 25OHD is about 22 days [214]. Thus, this lag period may represent the time taken to establish a new steady state.

Physical activity and gardening hours were positively correlated with 25OHD. The most likely explanation for this relationship is that time spent exercising or outdoors in the garden is a surrogate marker of sunshine exposure. Only a narrow band of UV-B light (290-315 nm) stimulates vitamin D synthesis, and this is effectively absorbed by glass and most plastics [180]. Levels of 25OHD are significantly lower in the housebound or hospitalised aged population, reflecting the decreased sunlight exposure in these patients [334]. In addition, there is a relationship between body weight and activity levels. People who exercise infrequently are more likely to have increased body weight, and therefore lower 25OHD levels.

Measures of fat mass, BMI, and body weight but not lean mass were independently and inversely related to 25OHD levels. Other researchers have previously reported that 25OHD levels are inversely related to fat mass in pre- and post-menopausal women [297, 301, 304] and older men and women [212]. To our knowledge, the relationship between 25OHD levels and fat mass in a large cohort of middle-aged and older men has not previously been reported. Other researchers have reported that 25OHD levels are also inversely related to weight or BMI [211, 212, 297, 298, 301-306], but as we observed, the relationships are weaker than those observed between 25OHD and levels of fat mass [212, 297, 304]. The inverse

relationship between 25OHD and fat mass has been attributed to increased sequestration of fat-soluble vitamin D in adipose tissue in obese individuals [301, 303]. Other possible explanations would be that overweight people have decreased exposure to sunlight because of their choice of clothing, or because of decreased exercise levels and mobility in heavier individuals, or as I explore in Chapter 10, due to alterations in DBP with obesity.

Age was negatively correlated with 25OHD in women, and there is a physiological basis to potentially explain this relationship. With ageing, the formation of vitamin D in the skin from its precursors is less efficient. Total body irradiation with UV light causes a four-fold greater increase in serum 25OHD levels in Caucasian young adults than in elderly subjects [335]. There is also an age-related decline in intestinal calcium absorption [336] which promotes secondary hyperparathyroidism and accelerated hepatic catabolism of 25OHD [311]. In addition, older people may receive less sunlight exposure because of frailty and reduced exercise levels, or for cultural and behavioural reasons. However, even in the elderly, cutaneous production of vitamin D remains substantial, and it is estimated that a 10-minute exposure of head and arms (unprotected) three times per week, would be sufficient to prevent vitamin D insufficiency in this population [337]. Interestingly, age was not a predictor of 25OHD levels in men. Other studies have reported conflicting results with respect to the change in 25OHD levels with age. 25OHD levels have been reported to decline with age in a population of men and women [211], to decline with age in women but not men [302], to decline with age after 50 years in women and after 70 years in men [320], and not to decline with age [329]. The mean age of our cohort of men was 57 years, and only 14% were aged over 70 years, whereas the mean age of our cohort of women was 73 years. We believe that age- and gender-related differences in behavioural factors such as time spent outdoors, type and amount of physical activity, and other cultural factors such as concern regarding skin changes with aging, concerns about skin cancer, and use of sunscreen may underpin the differences between the genders that we observed in the relationships between age and 25OHD, rather than any gender-related biological differences.

While we observed similar seasonal variations in 25OHD levels in men and women, men had higher 25OHD levels throughout the year. Gender differences in 25OHD levels have been reported in some [211, 302, 306, 318, 319, 338], but not all [339], previous studies. The reasons for such differences are not clear. Gender-related differences in the skin synthesis of vitamin D [211, 302] and cultural factors leading to difference in sun exposure between

genders [320] have been suggested as explanations. We did not record participants' sunlight exposure but did record time spent on physical activities, an indirect measure of this. While women spent more time gardening and men spent more time on other leisure pursuits and in employment, there was no difference in overall levels of physical activity between genders. It is possible, however, that women are more likely than men to exercise indoors, or to practise skin protection during exercise outdoors. Men aged greater than 60 years in Auckland have higher incidence of melanoma than women [340] which supports the latter hypothesis. Another possible explanation for the differences in 25OHD levels between genders is that women have greater amounts of body fat in which to sequester vitamin D. However, after adjusting for differences in body fat between men and women, the differences in 25OHD levels remained.

Since month of blood sampling, physical activity, and sunlight exposure are all indirect measures of exposure to UV-B, this suggests that there are only two major biological determinants of 25OHD levels— UV-B exposure and fat mass. The similarity in the strength and direction of the relationships between 25OHD and these variables for both genders suggests that these major biological determinants of 25OHD levels do not differ between men and women.

There are limitations to our studies. Men were not recruited at equal rates throughout the year with fewer men recruited during spring and more during summer. Although the study sample for the women was randomly selected from the New Zealand electoral roll, the relatively low response rate means that there is a risk of selection bias. Similarly, men were volunteers who responded to newspaper advertisements, again raising the possibility of selection bias. It may be that those who participated in the studies were more interested in health, and had healthier lifestyles than those who did not participate. The results may therefore be biased toward showing higher average levels of 25OHD than exist in the underlying population. On the other hand, those who did participate may already have concerns about their bone health and represent those with low vitamin D status. However, there is no reason to believe that the seasonal variation in measured parameters or the association between 25OHD levels and other measures would be any different in these study samples to the general population from which they are drawn. The interpretation of multiple statistical tests, as used here, must be undertaken with caution, since it is possible to detect significant associations between variables simply by performing multiple comparisons. However, the strong physiological

basis underlying these correlations implies that they are real, and not simply an artefact of multiple testing.

In summary, this cross-sectional study of healthy, independent-living, elderly, post-menopausal women and healthy, independent-living, middle-aged and older men has demonstrated that there is seasonal variation in vitamin D status, even in a subtropical area such as northern New Zealand. Of note, even in summer, a significant number of women were vitamin D insufficient, and this number increased substantially in winter months. In contrast, men have normal levels of 25OHD throughout the year with low rates of vitamin D insufficiency. In women, 25OHD levels were related to age, fat mass, and physical activity, with older, heavier, and less active women having lower serum levels of 25OHD. In men, 25OHD levels were related to fat mass and physical activity, but did not fall with age. Men at the greatest risk of vitamin D insufficiency were those who were dark-skinned, overweight, and sedentary. Although it is widely acknowledged that the elderly, institutionalised population often has suboptimal vitamin D status, this study demonstrates that healthy, independently-living, elderly women may also have vitamin D insufficiency. This study therefore lends support to the suggestion that vitamin D supplementation, particularly during winter, should become standard practice for elderly women, even in subtropical regions. In contrast, routine vitamin D supplementation for healthy older men is not warranted.



# **Chapter 9: The effects of seasonal variation of 25-hydroxyvitamin D and fat mass on diagnosis of vitamin D sufficiency**

## **Introduction:**

Vitamin D insufficiency in adults causes myopathy, osteopenia, secondary hyperparathyroidism, and osteomalacia [7]. The serum level of 25OHD is considered to be the best estimate of body stores of vitamin D [7]. Estimates of the level of serum 25OHD above which vitamin D stores are considered adequate vary widely, from 25 nmol/L to 100 nmol/L [186]. As discussed in Chapter 8, the two major biological determinants of 25OHD levels are UV-B exposure and fat mass. In countries distant from the equator, there is seasonal variation of UV-B levels due to the lower angle of the sun and greater cloud cover in winter months. More clothes are also worn in winter thereby reducing skin exposure to UV-B. As a result of this seasonal variation in UV-B, there is seasonal variation in 25OHD levels, such that levels are highest in late summer and early autumn and lowest in late winter and early spring. 25OHD levels are inversely associated with fat mass. This has been attributed to the sequestration into adipocytes of fat-soluble vitamin D generated in the skin or orally ingested, before it could be transported to the liver and converted to 25OHD [303]. In Chapter 8, I described seasonal variation in 25OHD levels in healthy, independent-living, middle-aged and older men and post-menopausal women living in Auckland, New Zealand (37°S). Such seasonal variation in 25OHD levels means that individuals could have adequate 25OHD levels in the summer and autumn months yet have suboptimal levels in winter and spring. We set out to determine the effects of seasonal variation of 25OHD on a pre-selected threshold level for diagnosis of vitamin D sufficiency (50 nmol/L) and whether fat mass or body weight modify these effects.

## **Methods:**

### ***Participants:***

1606 healthy, independent-living, post-menopausal women and 378 healthy, independent-living, middle-aged and older men volunteered for two separate studies of calcium supplementation. The protocols and methods for these studies have been discussed in detail in

Chapter 8. In brief, men aged at least 40 years, and women aged at least 55 years and more than 5 years postmenopausal were eligible to participate. Potential participants were ineligible if they had significant renal, hepatic or thyroid dysfunction, any other major ongoing disease, including malignancy, undiagnosed diabetes mellitus, or metabolic bone disease. Further exclusion criteria included the use of medication known to affect calcium metabolism, the use of cholecalciferol supplements in doses exceeding 1000 IU/day, the current use of glucocorticoids, or the use of testosterone, hormone replacement therapy, anabolic steroids, fluoride, or bisphosphonates in the previous one year. In addition, men were ineligible if they had coronary heart disease, an estimated five year cardiovascular risk of more than 15% [321], or were receiving therapy for hyperlipidaemia. Both studies received ethics approval from the Auckland Ethics Committee.

### ***Measurements:***

Height was measured at baseline using a Harpenden stadiometer, weight was recorded using electronic scales, and all participants supplied a fasting blood sample. Body composition was measured using a Lunar Prodigy DXA (GE Lunar, Madison, WI) in the men and a Lunar Expert DXA, software version 1.7 (GE Lunar, Madison, WI) in the women. Serum 25OHD was measured by RIA (DiaSorin, Stillwater, MN) in all the women and the first 252 men, then using a chemiluminescent assay (Nichols, San Juan Capistrano, CA) in the last 126 men. All 25OHD samples from both studies were measured in one laboratory which takes part in, and meets the performance targets for, the Vitamin D External Quality Assessment Scheme (DEQAS) [322]. Because there was a change in assay during the studies, we developed equations to allow the interconversion of results obtained from the two assays, as described in Chapter 8. All 25OHD measurements using the Nichols assay were converted to predicted DiaSorin values using the equation  $\text{DiaSorin} = \text{Nichols} * 0.75 + 5.6$ .

### ***Statistics:***

25OHD levels were plotted against the day of the year the blood sample was taken in each of the cohorts and a sine curve fitted. It was assumed that 25OHD levels throughout the year for each participant would follow a similar sine curve to the population. Therefore the sine curve for any individual would be the sine curve for the population translated along the y-axis until it intersected with the known 25OHD level on the known day of the year for that individual. By solving the equation for the sine curve for each individual, we were able to predict the

peak 25OHD level, the nadir 25OHD level and the number of days during which the 25OHD level was below the threshold for vitamin D sufficiency of 50 nmol/L. The equation for each sine curve was:  $25\text{OHD} = \text{baseline} + \text{amplitude} * \text{sine}(\text{angular frequency} * \text{day of year} + \text{phase shift})$ . The amplitude of the sine curve is the maximal deviation from the baseline  $[(\text{peak value} - \text{nadir value})/2]$ ; the angular frequency is  $2*\pi/\text{period}$  ( $2*\pi/365$ ); and the phase shift is the amount of translation along the x-axis.

To determine the effect of fat mass, we divided the cohorts into quartiles of fat mass and fitted a separate sine curve for each quartile of fat mass. We then used simple linear regression to compare the amplitude, peak, and nadir of the sine curves with the mean fat mass of each quartile. Linear regression was also used to compare fat mass and body weight to convert the effects seen for fat mass into comparable predicted effects for body weight. Finally, we repeated these analyses using body weight quartiles instead of fat mass quartiles.

All sine curve fitting and linear regression was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). All other statistical calculations were carried out using the SAS software package (SAS Institute, Cary, NC version 9.1). All tests were two-tailed and statistical significance was set at  $P < 0.05$ .

## **Results:**

The baseline characteristics of each cohort have been described in detail in Chapter 8 and are shown in Table 9.1.

**Table 9.1: Baseline characteristics of the study populations**

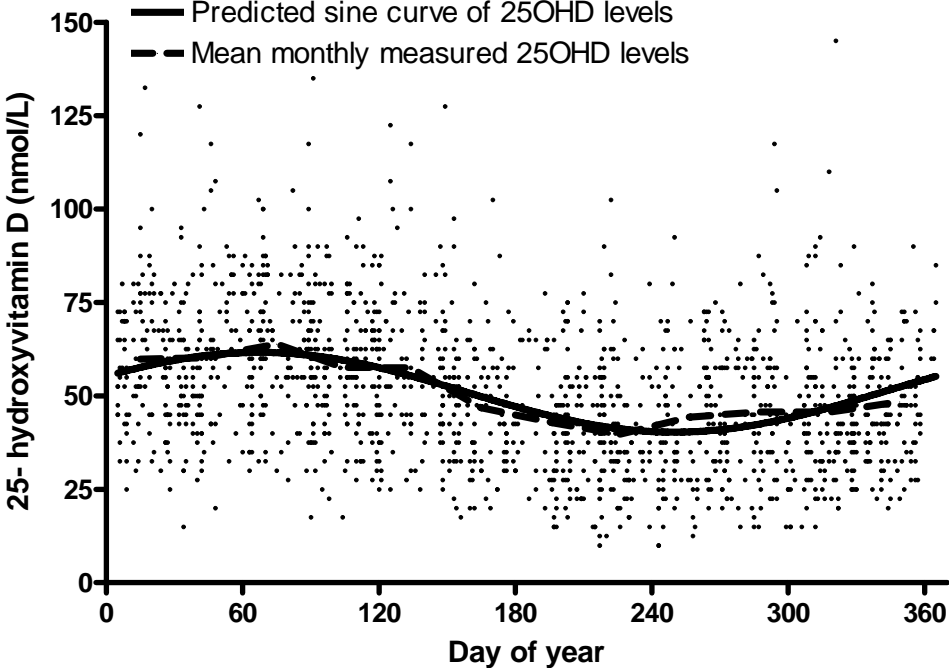
	<b>Men (N=378)</b>	<b>Women (N=1606)</b>
<b>Age (y)</b>	57 (11)	74 (4)
<b>Height (cm)</b>	176 (7)	159 (6)
<b>Weight (kg)</b>	82 (13)	67 (11)
<b>BMI (kg/m<sup>2</sup>)</b>	26.4 (3.4)	26.4 (4.3)
<b>Lean mass (kg)</b>	59 (7)	36 (4)
<b>Fat mass (kg)</b>	19 (8)	27 (10)
<b>Percent fat</b>	24 (7)	41 (8)
<b>25OHD (nmol/L)</b>	85 (31)	51 (19)

Data are mean (SD).

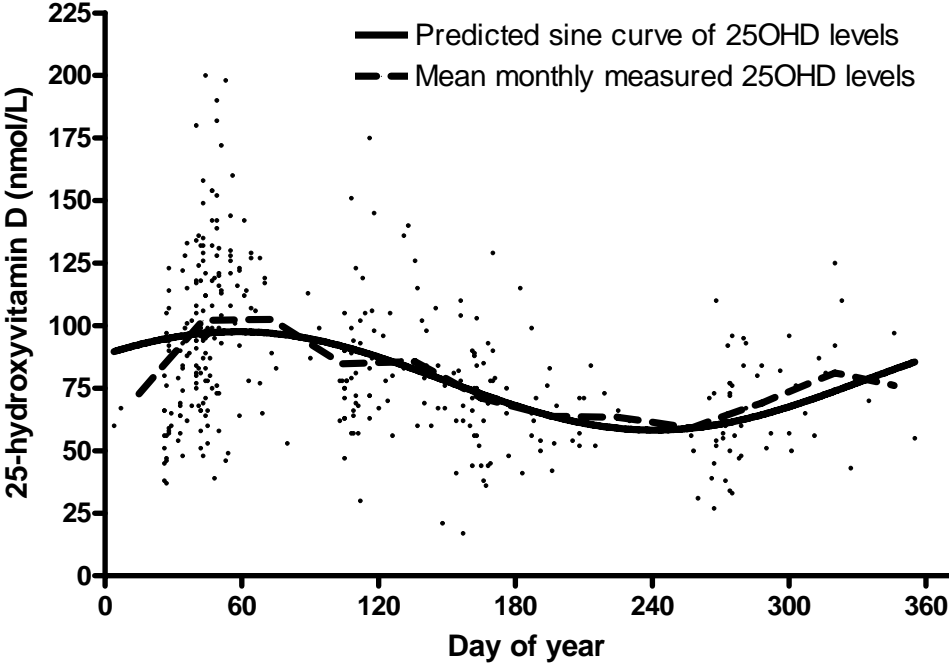
Figure 9.1 shows the sine curve fitted for the cohort of women. The equation for the fitted sine curve is  $y = 50.99 + 10.67 * \text{sine}(\text{frequency} * x + 0.41)$ ,  $r^2 = 0.15$ . Figure 9.2 shows the sine

curve fitted for the cohort of men. The equation for the fitted sine curve is  $y = 77.89 + 19.67 * \sin(\text{frequency} * x + 0.57)$ ,  $r^2 = 0.19$ . There was excellent agreement between the fitted sine curve and the mean monthly 25OHD levels in both cohorts (Figures 9.1 and 9.2).

**Figure 9.1: Sine curve of best fit for 25OHD in the cohort of women (N=1606) with measured mean monthly 25OHD for comparison**



**Figure 9.2: Sine curve of best fit for 25OHD in the cohort of men (N=378) with measured mean monthly 25OHD for comparison**



From the predicted sine curves, 73% of the women had a nadir 25OHD less than 50 nmol/L consistent with vitamin D insufficiency. In comparison, the observed prevalence of vitamin D insufficiency was 49%, ranging from 23-74% depending on the month of blood sampling, as described in Chapter 8. 39% of the men had a predicted nadir 25OHD less than 50 nmol/L, in comparison to an observed prevalence of vitamin D insufficiency of 9% (range 0-26%) as described in Chapter 8. In the 73% of women predicted to have nadir 25OHD less than 50 nmol/L, 28% were predicted to be vitamin D insufficient for the entire year, and the mean (SD) number of days with predicted vitamin D insufficiency was 250 (108). In the 39% of men predicted to have nadir 25OHD less than 50 nmol/L, 3% were predicted to be vitamin D insufficient for the whole year, and the mean (SD) number of days with predicted vitamin D insufficiency was 165 (89). From the sine curves, we determined the minimum 25OHD level for each month required to ensure that the 25OHD level was maintained at more than 50 nmol/L throughout the year (Table 9.2). During summer (December-March), this value approached 90 nmol/L in men, and 70 nmol/L in women.

**Table 9.2: The minimum 25OHD level required to have a predicted nadir 25OHD more than 50 nmol/L, by month of measurement**

<b>Month of Year</b>	<b>Men</b>	<b>Women</b>
January	81	65
February	87	69
March	87	71
April	79	67
May	69	62
June	59	57
July	52	52
August	50	50
September	50	50
October	53	51
November	61	54
December	71	60

To determine the effect of fat mass on seasonal variation of 25OHD, we divided the cohorts into quartiles of fat mass and fitted a sine curve to each quartile. The peak 25OHD level and amplitude of the sine curves decreased with increasing fat mass in both men and women while the nadir 25OHD levels were similar (Table 9.3). Thus, subjects in the highest quartile of fat mass had smaller seasonal excursions in 25OHD levels and lower peak 25OHD levels than subjects in the lowest quartile of fat mass (Figure 9.3). In women, each 1 kg difference in fat mass was associated with a change in peak 25OHD of -0.52 nmol/L, nadir 25OHD of -0.05 nmol/L, and amplitude of -0.23 nmol/L. We used linear regression to compare fat mass and body weight in this cohort of women and found that each increase in fat mass of 1 kg was

associated with an increase in body weight of 1.12 kg. Thus, each 1 kg difference in body weight was associated with a change in peak 25OHD of -0.46 nmol/L, nadir 25OHD of -0.04 nmol/L, and amplitude of -0.21 nmol/L. When we repeated the analyses of peak 25OHD levels and seasonal change in 25OHD using weight quartiles instead of fat mass quartiles, we obtained similar results. In men, each 1 kg difference in fat mass was associated with a change in peak 25OHD of -1.3 nmol/L, nadir 25OHD of -0.27 nmol/L, and amplitude of -0.51 nmol/L. Each increase in fat mass of 1kg was associated with an increase in body weight of 1.33 kg. Thus, in men each 1 kg difference in body weight was associated with a change in peak 25OHD of -0.97 nmol/L, nadir 25OHD of -0.20 nmol/L, and amplitude of -0.38 nmol/L. When we repeated these analyses using weight quartiles instead of fat mass quartiles we obtained similar results. There was no association between fat mass quartile and the number of days/year of predicted vitamin D insufficiency in men or women.

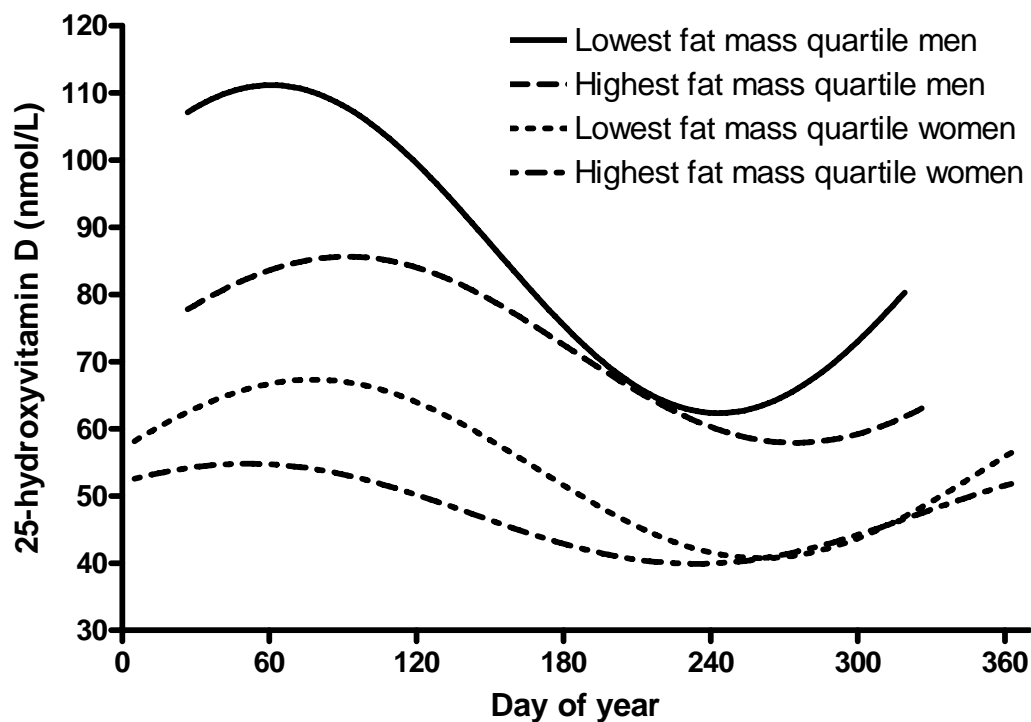
**Table 9.3: The effect of fat mass on seasonal variation of 25OHD levels**

Quartile	1st	2nd	3rd	4th	P
<b>Men</b>					
Mean fat mass (kg)	10.4	16.3	20.7	29.3	
Peak 25OHD(nmol/L)	111 (102-120)	101 (91-110)	100 (91-110)	86 (78-93)	0.025
Nadir 25OHD(nmol/L)	62 (46-78)	61 (44-78)	56 (42-70)	58 (47-69)	0.29
Amplitude (nmol/L)	24 (14-35)	20 (9-31)	22 (13-32)	14 (6-22)	0.12
<b>Women</b>					
Mean fat mass (kg)	16.0	23.2	29.1	39.6	
Peak 25OHD(nmol/L)	67 (64-70)	63 (60-66)	61 (59-64)	55 (52-58)	0.008
Nadir 25OHD(nmol/L)	41 (37-44)	42 (39-45)	42 (39-45)	40 (37-43)	0.52
Amplitude (nmol/L)	13 (11-16)	10 (8-13)	10 (8-12)	7 (5-10)	0.027

Data are mean (95% CI). P for the slope of the regression line for mean values of peak 25OHD, nadir 25OHD or amplitude versus quartile of fat mass being different from 0.

Finally, to determine whether the differences between the amplitudes and the peak and nadir values of the sine curves in men and women were due to differences in fat mass between genders, we restricted our analyses to men ( $N=162$ ) and women ( $N=725$ ) of the same fat mass range (15-25 kg). We found that the amplitudes and the peak and nadir values of the sine curves for these subgroups were similar to the values for the entire cohorts. Thus, the sine curve for the subgroup of men had larger amplitude (19 nmol/L), peak value (96 nmol/L), and nadir value (58 nmol/L) than the curve for the subgroup of women (amplitude 11 nmol/L, peak 64 nmol/L, nadir 42 nmol/L), suggesting that fat mass differences between genders could not fully explain the gender differences in the amount of seasonal variation of 25OHD.

**Figure 9.3: The effect of fat mass on seasonal variation of 25OHD levels in men and women**



Data are shown from the lowest and highest quartiles only for clarity.

### **Discussion:**

Seasonal variation in 25OHD levels has a substantial impact upon diagnosis of vitamin D insufficiency. We found that 49% (range: 23-74%) of post-menopausal women and 9% (range: 0-26%) of middle-aged and older men were vitamin D insufficient in the month of measurement. However, the current analysis predicts much higher prevalences of vitamin D insufficiency (73% in women and 39% in men) in late winter/early spring. In those people predicted to have vitamin D insufficiency, the mean predicted duration was 250 days/year in women and 165 days/year in men. Thus, many people are predicted to have suboptimal 25OHD levels for a substantial proportion of the year despite having apparently adequate levels at the time of testing. We found that 25OHD levels in summer and autumn of at least 70-90 nmol/L in men and 60-70 nmol/L in women are required to ensure vitamin D sufficiency throughout the year. Currently, there is no universally accepted definition of vitamin D sufficiency, and estimates of 25OHD levels considered to be adequate range from as low as 25 nmol/L to as high as 100 nmol/L, with the most commonly recommended

thresholds between 50-80 nmol/L [186]. The need to consider seasonal variation when interpreting these thresholds has not been widely discussed. Although not stated explicitly, it can be inferred that definition of vitamin D sufficiency refers to the lowest 25OHD level during the year. Vitamin D insufficiency is associated with adverse effects such as secondary hyperparathyroidism, increased bone turnover, decline in bone density, and increased risk of fractures [7, 186]. Population studies have reported higher rates of bone remodelling [187, 189] and fragility fracture [189, 341, 342] during winter, suggesting that any period of vitamin D insufficiency is undesirable. Thus, in locations where seasonal variation of 25OHD occurs, thresholds for diagnosis of vitamin D sufficiency will also vary by season.

Seasonal variations of 25OHD levels have been widely reported, even in subtropical locations with year-round sunny weather [343]. In our cohorts, who reside in a subtropical location at latitude 37°S, there were seasonal variations in 25OHD of 21 nmol/L (peak 25OHD-nadir 25OHD) in women and 39 nmol/L in men. The amount of seasonal variation of 25OHD is likely to be determined by the latitude and the climate. In countries at higher latitudes, lower angles of incidence of incoming solar radiation during winter result in UV rays travelling a greater distance through the atmosphere and therefore increased atmospheric absorption of UV radiation. Seasonal changes in cloud cover may also contribute to the increased atmospheric absorption of UV radiation. In addition, exposure of the skin to UV-B is generally decreased during the colder months because more clothes are worn. Thus, adjustment for the effects of seasonal variation on thresholds for diagnosis of vitamin D sufficiency needs to be individualised to the latitude and climate of a location.

The effect of fat mass on seasonal variation of 25OHD levels has not been previously reported. As discussed in Chapters 7 and 8, fat mass is a small but important determinant of 25OHD levels in cross-sectional analyses [211, 212, 297, 298, 301-305], in which fat mass and 25OHD levels are inversely associated even after adjustment for potential confounding factors such as exercise levels, which are a surrogate measure of sunlight exposure. This relationship may occur because vitamin D and its metabolites are fat soluble, leading to increased sequestration in adipose tissue in obese individuals [301, 303]. Other possible explanations would be that overweight people have decreased exposure to sunlight because of their choice of clothing or because of decreased exercise levels and mobility. Low 25OHD levels promote secondary hyperparathyroidism [7] which may in turn increase hepatic catabolism of 25OHD, thereby further lowering 25OHD levels [311]. Our findings that



greater fat mass was associated with lower peak 25OHD levels and smaller seasonal variation of 25OHD, but no change in nadir 25OHD levels, are consistent with an effect of both reduced sunlight exposure in heavier individuals, and adipose tissue acting as a reservoir of vitamin D and its metabolites. Regardless of fat mass, reduced exposure to sunlight will lead to lower peak 25OHD levels. If adipocytes act as a reservoir of vitamin D and its metabolites and buffer against both higher 25OHD levels in summer and lower levels in winter, then increased adiposity would be associated with reduced peak 25OHD levels, reduced seasonal variation, and higher than expected nadir 25OHD levels. A combination of these two hypotheses, both reduced sunlight exposure and an increased reservoir of vitamin D and its metabolites, would therefore explain our findings.

Fat mass modifies the effect of seasonal variation on thresholds for diagnosis of vitamin D sufficiency. People in the highest quartile of fat mass have lower peak 25OHD levels and smaller amounts of seasonal variation of 25OHD levels in comparison to people in the lowest quartile of fat mass, while nadir 25OHD levels are similar across fat mass quartiles. Thus, seasonally-adjusted thresholds for diagnosis of vitamin D sufficiency decrease with increasing fat mass, and increase with decreasing fat mass. However, this effect is small and may only be clinically relevant for people whose body weight or fat mass is greater than one standard deviation from the mean and who have 25OHD measurements taken in summer. The differences in 25OHD levels and seasonally-adjusted thresholds for diagnosis of vitamin D sufficiency between men and women are not fully explained by the differences in fat mass between genders. As discussed in Chapter 8, it is likely that gender-related differences in behavioural and cultural factors associated with sunlight exposure, physical activity, and skin protective practices and/or gender-related differences in vitamin D metabolism [211, 302] underpin the differences between men and women in 25OHD levels and seasonally-adjusted thresholds for diagnosis of vitamin D sufficiency.

There are limitations to our study. Participants were healthy independent volunteers and were predominantly of European descent. The findings therefore may not be applicable to people with pigmented skin, to different population groups such as the frail elderly, or to people living in different latitudes or different climates.

In summary, we found that seasonal variation of 25OHD levels has significant impact upon thresholds for diagnosis of vitamin D sufficiency. Levels of fat mass affect peak 25OHD levels and the amount of seasonal variation of 25OHD, but not nadir 25OHD levels, and

potentially impact upon thresholds for diagnosis of vitamin D sufficiency. Since seasonal variation of 25OHD is primarily dependent on latitude and climate, seasonally-adjusted thresholds for diagnosis of vitamin D sufficiency will need to be individualised for different locations. Clinicians should take into account the season of sampling when determining whether a patient is at risk of vitamin D insufficiency during the year.

## **Chapter 10: Age-, gender-, and weight-related effects on levels of 25-hydroxyvitamin D are not mediated by vitamin D binding protein**

### **Introduction:**

As discussed in Chapter 5, vitamin D is formed in the skin by the photolysis of steroid precursors by UV-B radiation. Newly formed vitamin D is bound to DBP and transported to the liver where it is hydroxylated to 25OHD. 25OHD is further hydroxylated in the kidney to 1,25(OH)<sub>2</sub>D, the most active metabolite of vitamin D [180]. Both 25OHD and 1,25(OH)<sub>2</sub>D are tightly bound to DBP in the serum. Only 0.02%-0.05% of 25OHD and 0.2%-0.6% of 1,25(OH)<sub>2</sub>D exist as free or unbound forms in serum [344]. Currently available immunoassays for 25OHD and 1,25(OH)<sub>2</sub>D measure both DBP-bound and unbound sterol, and are potentially therefore affected by changes in levels of DBP.

In Chapter 8, we reported inverse relationships of total 25OHD with fat mass in men and women, and age in women, and gender differences in total 25OHD levels. Other researchers have reported similar relationships between 25OHD and body weight, fat mass and age [211, 212, 297, 298, 301-306, 320], and gender differences in total 25OHD levels [211, 302, 306, 318, 319, 338]. It has been suggested that the relationship between total 25OHD and fat mass is due to increased sequestration of the fat soluble vitamin D in adipose tissue [303]. The age and gender differences in total 25OHD levels have been attributed to a reduction in ability to synthesise vitamin D in the skin with aging [335], and differences in sun exposure due to behavioural and cultural differences between genders [211, 302, 320], as described in Chapter 8. Observational studies have reported that total 25OHD levels are reduced in a number of conditions including diabetes mellitus [345], ischaemic heart disease [346], congestive heart failure [347], hypertension [348], and a variety of cancers [349]. Since these conditions are all associated with increased body weight [265, 287], adiposity may be an important confounder of these associations. Additionally, it has been suggested that vitamin D deficiency may be associated with an increased risk of type I diabetes, multiple sclerosis, and rheumatoid arthritis [184].

Circulating concentrations of sex-hormone binding globulin, another steroid hormone binding protein, are inversely related to body weight, perhaps mediated via an effect of

hyperinsulinaemia on its hepatic synthesis, and are positively associated with oestradiol levels [350]. Thyroxine binding globulin levels also increase with orally administered oestrogen and in pregnancy [351]. Total 25OHD levels are higher in women who take oral contraceptives or hormone replacement therapy containing oestrogen [352-354], and DBP levels rise in response to the use of oestrogen [352, 353]. Therefore, we hypothesised that the relationships between total 25OHD and fat mass, age, gender and the conditions described previously may be mediated by variation of DBP levels, a question that does not appear to have been addressed previously.

## **Methods:**

### ***Participants:***

50 men and 50 women were randomly selected from subjects volunteering to be involved in studies of calcium metabolism in normal subjects. The men were at least 40 years of age and the women were post-menopausal and at least 55 years of age. Participants did not have significant chronic medical conditions and were not taking medications likely to impact on calcium or vitamin D metabolism, including oestrogen and bisphosphonates. None were taking vitamin D supplements in doses greater than 1000 IU/day. The study had local ethics committee approval and the subjects gave written informed consent.

### ***Measurements:***

Height was measured at baseline using a Harpenden stadiometer, weight was recorded using electronic scales, and all participants supplied a fasting blood sample. BMD of the lumbar spine, proximal femur and total body was measured using a Lunar Prodigy DXA (GE Lunar, Madison, WI). Body composition was derived from the whole body analysis. Serum total 25OHD was measured by RIA (DiaSorin, Stillwater, MN) in the 50 men and using a chemiluminescent assay (Nichols, San Juan Capistrano, CA) in the 50 women. All 25OHD samples were measured in one laboratory which takes part in, and meets the performance targets for, the Vitamin D External Quality Assessment Scheme (DEQAS) [322]. The interassay CV for the DiaSorin assay was 8.8% and for the Nichols assay was 7.5% in our laboratory. Because there was a change in assay during the studies, we developed equations to allow the interconversion of results obtained from the two assays, as described in Chapter 8.

All data obtained using the Nichols assay were converted to predicted DiaSorin results using the derived equation  $\text{DiaSorin} = \text{Nichols} * 0.75 + 5.6$ .

DBP was measured by single radial immunodiffusion [355]. Free 25OHD levels were calculated using the following equation:

$$[\text{Free 25OHD}] = [\text{Total 25OHD}] / (1 + K_{a\text{ alb}} * [\text{albumin}] + K_{a\text{ DBP}} * [\text{DBP}])$$

where  $K_{a\text{ alb}}$  and  $K_{a\text{ DBP}}$  are the affinity constants for albumin and DBP respectively ( $K_{a\text{ alb}} = 6 * 10^5 \text{ L/mol}$ ,  $K_{a\text{ DBP}} = 7 * 10^8 \text{ L/mol}$ ) [356]. Percentage free levels were calculated as the ratio of free 25OHD to total 25OHD \* 100.

### **Statistics:**

Student's t-test was used to assess differences in DBP levels between genders. Pearson correlation analysis was used to test for significant associations between DBP, total 25OHD, free 25OHD and the other variables measured. Multiple linear regression was used to test for relationships between DBP and other variables after adjusting for gender. The statistical significance level was set at  $P < 0.05$  and all tests were two-tailed. All statistical analyses were obtained using the SAS software package (SAS Institute, Cary, NC version 9.1).

### **Results:**

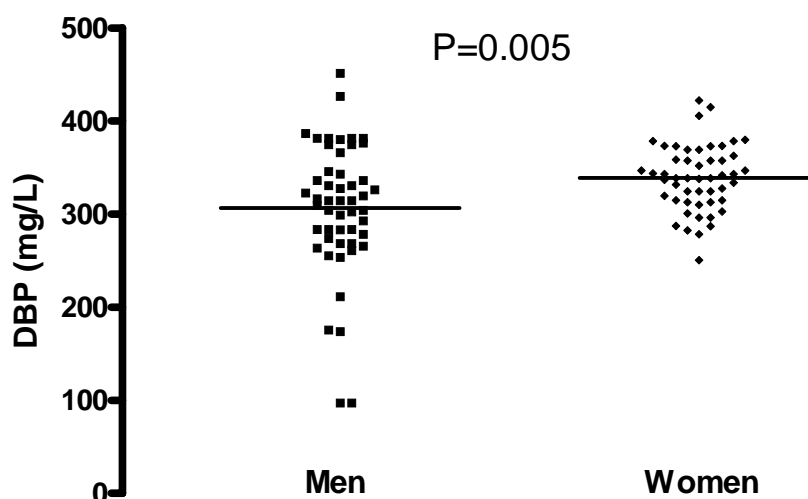
Biochemical and anthropometric characteristics of the study groups are shown in Table 10.1. Mean (SD) DBP levels were significantly higher in women [339 (36) mg/L] than men [307 (71) mg/L],  $P = 0.005$  (Figure 1), and total serum 25OHD levels were significantly lower in women [67 (23) nmol/L] than men [91 (39) nmol/L],  $P < 0.001$ . Because of these differences and the differences in age and body composition between genders, we analysed the two groups separately. Pearson's correlations between total 25OHD, free 25OHD, DBP and other variables from Table 10.1 are shown in Table 10.2.

DBP levels were inversely related to the percent free 25OHD, as would be expected from the method of its derivation. In women, there were moderately strong positive correlations between DBP and total 25OHD, and DBP and albumin, whereas in men, there were no significant correlations between DBP and any other variables measured. The correlations between DBP and measures of body weight and adiposity were negative in men and positive in women but did not reach statistical significance in either group. There were weak negative correlations between DBP and age in both men and women that did not reach statistical

**Table 10.1: Biochemical and anthropometric characteristics of the study groups**

Variable	Men (N=50)		Women (N=50)		P
	Mean (SD)	Range	Mean (SD)	Range	
Total 25OHD (nmol/L)	91 (39)	36-200	67 (23)	31-131	<0.001
DBP (mg/L)	307 (71)	97-452	339 (36)	251-422	0.005
Free 25OHD (pmol/L)	23 (10)	8-46	15 (5)	6-24	<0.001
Percent free 25OHD	0.026 (0.009)	0.017-0.065	0.023 (0.002)	0.018-0.030	0.008
Age (years)	55.6 (9.3)	38-77	67.5 (8.6)	51-85	<0.001
Weight (kg)	82.0 (11.1)	63-123	69.6 (12.8)	49-108	<0.001
Height (cm)	176.6 (6.1)	164-191	160.6 (6.0)	149-176	<0.001
BMI (kg/m <sup>2</sup> )	26.2 (3.1)	20-38	26.9 (4.6)	19-41	0.38
Fat mass (kg)	18.8 (7.0)	5-42	26.9 (9.4)	12-56	<0.001
Truncal fat mass (kg)	10.9 (4.3)	3-24	13.6 (5.4)	6-31	0.014
Lean mass (kg)	59.1 (6.4)	47-75	39.8 (5.1)	30-51	<0.001
Percent fat	23.7 (6.3)	9-37	39.4 (7.4)	25-54	<0.001
Adjusted serum calcium (mmol/L)	2.31 (0.08)	2.10-2.53	2.39 (0.07)	2.26-2.53	<0.001
Albumin (g/L)	44 (2)	38-50	43 (3)	37-51	0.03
Serum creatinine (mmol/L)	0.09 (0.01)	0.07-0.12	0.08 (0.01)	0.05-0.10	<0.001
Fasting glucose (mmol/L)	5.1 (0.5)	4.3-6.7	4.8 (0.7)	3.5-7.3	0.005
L1-4 BMD (g/cm <sup>2</sup> )	1.19 (0.14)	0.91-1.50	1.13 (0.20)	0.94-1.98	0.05
Total hip BMD (g/cm <sup>2</sup> )	1.03 (0.13)	0.79-1.31	0.96 (0.12)	0.73-1.43	0.004
Total body BMD (g/cm <sup>2</sup> )	1.23 (0.09)	1.08-1.42	1.11 (0.10)	0.94-1.48	<0.001

**Figure 10.1: DBP levels in men and women**



The horizontal lines represent the mean BMD for each group at that site.

**Table 10.2: Pearson's correlations between DBP, 25OHD, and other variables**

	Men (N=50)			Women (N=50)		
	DBP	Total 25OHD	Free 25OHD	DBP	Total 25OHD	Free 25OHD
<b>Total 25OHD</b>	0.18	1.00	0.80 <sup>b</sup>	0.34 <sup>a</sup>	1.00	0.95 <sup>b</sup>
<b>Free 25OHD</b>	-0.42 <sup>b</sup>	0.80 <sup>b</sup>	1.00	0.05	0.95 <sup>b</sup>	1.00
<b>Percent free 25OHD</b>	-0.91 <sup>b</sup>	-0.20	0.40 <sup>b</sup>	-0.99 <sup>b</sup>	-0.33 <sup>a</sup>	-0.04
<b>Age</b>	-0.25	0.07	0.24	-0.08	0.14	0.19
<b>Height</b>	0.15	0.10	0.00	-0.16	-0.01	0.06
<b>Weight</b>	0.24	-0.09	-0.22	-0.16	-0.37 <sup>b</sup>	-0.36 <sup>a</sup>
<b>BMI</b>	0.18	-0.16	-0.25	-0.11	-0.40 <sup>b</sup>	-0.41 <sup>b</sup>
<b>Lean mass</b>	0.26	0.01	-0.16	-0.14	-0.17	-0.14
<b>Fat mass</b>	0.13	-0.17	-0.22	-0.14	-0.41 <sup>b</sup>	-0.41 <sup>b</sup>
<b>Truncal fat mass</b>	0.09	-0.20	-0.21	-0.11	-0.44 <sup>b</sup>	-0.47 <sup>b</sup>
<b>Percentage fat</b>	0.04	-0.15	-0.15	-0.09	-0.38 <sup>b</sup>	-0.40 <sup>b</sup>
<b>Adjusted serum calcium</b>	-0.08	-0.02	0.00	-0.05	0.04	0.05
<b>Albumin</b>	-0.01	0.10	0.02	0.33 <sup>a</sup>	0.12	0.01
<b>Serum creatinine</b>	0.04	0.06	0.01	-0.04	0.33 <sup>a</sup>	0.35 <sup>a</sup>
<b>Fasting glucose</b>	0.18	0.05	-0.06	-0.11	0.03	0.07
<b>L1-4 BMD</b>	-0.10	0.08	0.11	-0.16	-0.09	-0.05
<b>Total hip BMD</b>	0.03	0.08	0.01	0.02	-0.08	-0.11
<b>Total body BMD</b>	0.08	0.08	-0.03	0.06	-0.06	-0.09

<sup>a</sup> P < 0.05, <sup>b</sup> P < 0.01

significance. When the groups of men and women were analysed together with gender included as a covariate, there was no relationship between DBP and age, weight or other measures of adiposity. Because there was a significant age difference between the groups of men and women, we restricted the analyses to men (N=22) and women (N=34) of similar age (range 55-71 years, mean age for men 62 years, for women 63 years). We found similar correlations between DBP and other variables measured in these subgroups of men and women compared to the correlations for each entire group.

### **Discussion:**

We found that DBP levels were 10% higher in women than in men, but that there was no relationship between DBP and age or measures of adiposity in men or women. There were positive correlations between DBP and total 25OHD levels in both men and women. Since women had higher levels of DBP than men, they would be predicted to have higher levels of total 25OHD. However, most studies that have reported gender differences in total 25OHD levels have reported higher levels in men than women [211, 302, 306, 318, 319, 338], as we observed in this study and in Chapter 8. The conflicting strength and direction of the

correlations between DBP and measures of body weight and adiposity suggests that there is no biological relationship between DBP and these variables. DBP was weakly inversely related to age. Thus, with aging DBP levels may fall slightly which in turn may contribute to lower total 25OHD levels. The strength of the correlations suggests that this effect, if present, is unlikely to be of clinical significance. Overall we found no evidence to support the hypothesis that the changes in total 25OHD levels with age, gender, or fat mass are due to underlying relationships between DBP and these variables.

Our results are similar to those of a recently reported study of DBP levels in 211 men aged 71-86 years [357]. In that study, there was no relationship between DBP and total 25OHD, but there were weak correlations between DBP and weight, BMI and fat mass. The correlations ( $r=0.19-0.21$ ) were similar to those in our study, but, in contrast to our findings, were statistically significant perhaps because of the larger sample size. The authors suggested that variation in DBP levels may in part explain the relationship between total 25OHD and fat mass. However, a positive association between fat mass and DBP would be predicted to mediate a positive relationship between total 25OHD and fat mass rather than the inverse relationship that we found in Chapter 8, and that others have reported [212, 297, 301, 304]. Our finding that the direction of the relationships between DBP and measures of weight or fat mass differs according to gender, when an inverse relationship exists between total 25OHD and body weight in both genders, suggests that weight-associated changes in DBP do not explain the relationship between total 25OHD and body weight.

Total 25OHD levels were negatively correlated with measures of adiposity in both men and women. Although the correlations were only statistically significant for women, the strength of the correlations in men in this substudy were similar to those from a larger cohort of 378 men described in Chapter 8, where the correlations were statistically significant. Compared to total 25OHD levels, calculated free 25OHD levels were similarly correlated with measures of adiposity in women, and more strongly correlated with the same variables in men. The relationship between total 25OHD and fat mass has been attributed to increased sequestration of fat soluble vitamin D in adipocytes. Worstman et al. [303] reported that obese individuals have similar serum vitamin D<sub>3</sub> levels but lower total 25OHD levels than non-obese individuals. Total body irradiation led to smaller increases in serum vitamin D<sub>3</sub> in obese individuals than non-obese individuals despite similar cutaneous percentage conversion of pre-vitamin D<sub>3</sub> to vitamin D<sub>3</sub>. Oral ingestion of vitamin D<sub>2</sub> led to lower peak serum vitamin



D<sub>2</sub> levels in obese compared to non-obese individuals. The authors therefore hypothesised that vitamin D generated in the skin or orally ingested was sequestered into adipocytes before it could be transported to the liver and converted to 25OHD [303]. Other researchers have found low serum vitamin D [358], and low serum 1,25(OH)<sub>2</sub>D [297] levels in association with obesity, suggesting that vitamin D and all its metabolites may be sequestered into adipocytes. It is not known whether the adipocytes simply store vitamin D or actively catabolise it.

Total 25OHD levels have also been reported to be lower in women than men [211, 302, 306, 318, 319, 338] and to decline with age [211, 302, 320]. In Chapter 8, we reported that the major determinants of total 25OHD levels are UV-B exposure and adiposity in both men and women. While a reduction in ability to synthesise vitamin D in the skin occurs with aging [335], the most likely explanation for the reported relationships of total 25OHD levels with gender and age is that there are differences in cultural and behavioural factors between genders and with aging that influence sunlight exposure, as discussed in Chapter 8. The findings of the current study, which demonstrate higher DBP levels, but lower total 25OHD levels, in women than men, argue against a role for DBP in the gender-associated differences in total 25OHD.

Vitamin D insufficiency, low vitamin D intake, and reduced total 25OHD levels have been associated with a variety of ailments including autoimmune conditions (type I diabetes, multiple sclerosis, and rheumatoid arthritis), some cancers, ischaemic heart disease and congestive heart failure, and components of the metabolic syndrome (insulin resistance, diabetes mellitus, and hypertension) [184, 345-349, 359]. Body weight and adiposity are important potential confounding factors for many of these conditions [265, 287]. We hypothesised that the increased body weight associated with many of these conditions may lead to lower DBP levels and thus decreased total 25OHD levels. Although the results of our study suggest that DBP does not mediate this relationship, increased fat mass may still account for some of these findings. Some of the studies reporting association between low total 25OHD levels and disease have attempted to control for body weight or body mass index. However, since the strongest relationship is between total 25OHD levels and percent body fat, as described in Chapter 8 and reported by others [304], and the relationship between body weight and percent body fat is non-linear, such adjustments may not be sufficient to exclude confounding by adiposity.

This study has several limitations. We have performed multiple statistical tests, and there is a possibility that some of the correlations which are statistically significant have arisen due to chance. The size of each group means that there was 80% power (at the 5% significance level) to detect univariate correlations of at least 0.38. Thus there is the possibility of a Type II error where some correlations have been misclassified as not statistically significant. Any such correlations are likely to be weak, accounting for less than 15% of the variance concerned. The possibility of selection bias also arises. Participants in this study were volunteers who may have chosen to take part because they were more aware of health issues and healthier than average, or, conversely, they may have chosen to take part because they believed they were at higher risk of osteoporosis because of family history or background of osteoporosis or poor health. There were differences in mean age between the groups of men and women and therefore the groups were not directly comparable. However, when we restricted our analyses to comparably aged men and women, the relationships between DBP and the other variables measured were not different to those in the entire cohort.

In summary, we found no evidence to support the hypothesis that DBP levels are related to age, or adiposity. Women had slightly higher levels of DBP than men but this does not explain the reported gender differences in total 25OHD levels. Our data therefore suggest that changes in total 25OHD levels with age, gender, or fat mass are not due to underlying relationships between DBP and these variables, and that the consistently observed relationships of total 25OHD with body composition have a biological origin other than adaptation to plasma transport.

## Conclusions to Chapters 5-10:

We meta-analysed all published studies of patients with PHPT and a comparable age-matched control group that reported body weight or BMI and found that people with PHPT were on average 3.1-3.3 kg heavier than controls. PHPT may directly cause increased body weight or, alternatively, it may occur as a consequence of a prolonged duration of increased body weight. Data from very large prospective cohort studies suggest that an increased body weight of this magnitude would be associated with an increased prevalence of a number of diverse conditions that have been previously associated with PHPT. Thus, our observations suggest a potential explanation for these “non-classical” associations of PHPT. They also suggest that the indications for parathyroidectomy in patients with asymptomatic disease should be re-examined since many editorialists recommend parathyroidectomy in an attempt to reduce the risk of developing these “non-classical” complications. Subsequently, we found that body weight, and more specifically fat mass, is positively related to serum levels of PTH in healthy eucalcaemic post-menopausal women and that this relationship was independent of vitamin D insufficiency and the inverse association between fat mass and 25OHD. Since increased body weight appears to precede the development of PHPT, this suggests that increased body weight, increased fat mass and obesity may predispose to the development of PHPT.

We found that there were seasonal variations in vitamin D status in healthy, independent-living, elderly, post-menopausal women and middle-aged and older men, but that men had higher levels of 25OHD and lower rates of vitamin D insufficiency throughout the year than women. Thus, in summer a significant number of women were vitamin D insufficient, and this number increased substantially in winter, whereas in men there were low rates of vitamin D insufficiency throughout the year. In women and men, the major determinants of serum 25OHD levels were surrogate measures of UV-B exposure and fat mass. Those people at greatest risk for vitamin D insufficiency are those with limited sun exposure, and those who are dark skinned, sedentary and overweight. Based on our data, vitamin D supplementation, particularly during winter, should become standard practice for elderly women in Auckland, whereas routine vitamin D supplementation for healthy older men is not warranted. However, these conclusions may not apply to different populations such as men with co-morbidities or risk factors for vitamin D insufficiency.

25OHD levels were higher in men than women, declined with age in women but not men, and declined with increasing body weight or fat mass. We hypothesised that the relationships

between 25OHD and these variables may be mediated by variation of DBP levels. However, we found no evidence in a cross-sectional analysis of older men and women to support this hypothesis. It is likely that the relationship between 25OHD and fat mass arises because vitamin D and its metabolites are fat soluble and sequestered into adipocytes. The most likely explanation for the relationships between 25OHD and gender and age is that there are differences in cultural and behavioural factors between genders and with aging that influence sunlight exposure.

Since 25OHD levels vary by season in response to UV-B exposure, individuals could have adequate 25OHD levels in the summer and autumn months yet have suboptimal levels in winter and spring. We found that seasonal variation of 25OHD levels has significant impact upon thresholds for diagnosis of vitamin D sufficiency. In addition, the amount of fat mass affects peak 25OHD levels and the amount of seasonal variation of 25OHD, but not nadir 25OHD levels, and potentially impacts upon thresholds for diagnosis of vitamin D sufficiency. We showed that significant numbers of women and men would be erroneously misclassified as vitamin D sufficient if the time of the year and fat mass are not considered in determining whether a patient is at risk of vitamin D insufficiency throughout the year. Seasonally-adjusted thresholds for determination of vitamin D sufficiency need to be individualised depending on the latitude and climate of the location.

Body weight, and more specifically, fat mass is a major determinant of serum PTH and serum 25OHD levels and appears to have key roles in the pathogenesis of PHPT and vitamin D insufficiency. Both of these conditions are common and important causes of secondary osteoporosis. While increasing fat mass may promote the development of PHPT or vitamin D insufficiency, increasing body weight and fat mass are also associated with increasing BMD. Further research is required to determine why increasing fat mass sometimes promotes osteoporosis (mediated by PHPT and vitamin D insufficiency) whereas it generally tends to reduce the likelihood of osteoporosis.

There are many options for future research in these areas. Further observational studies of patients with asymptomatic PHPT are required to determine whether increased body weight accounts, at least in part, for some or all of the non-classical associations of primary hyperparathyroidism. Similarly longitudinal studies of calcitropic hormones in post-menopausal women may provide insights into the relationship between body fat and PTH, and the possible causal relationship between obesity and PHPT. Further research is required to

determine the optimal circulating serum 25OHD level, and longitudinal studies of participants with vitamin D insufficiency may provide evidence for such a threshold. We also hope to validate the models of the seasonal changes in the thresholds for vitamin D insufficiency developed in Chapter 9 in unselected measurements of 25OHD from our hospital.

At first glance, the three conditions causing secondary osteoporosis that I have discussed in this thesis – PHPT, vitamin D insufficiency, and HIV infection – appear to have little in common. However, as discussed previously in the conclusions to Chapter 1-4 and in the current section, it is clear that body weight and/or fat mass have a crucial role in the pathogenesis of osteoporosis in association with HIV, and the pathogenesis of PHPT and vitamin D insufficiency which in turn predispose to osteoporosis. In fact, HIV-related osteoporosis may be predominantly caused by the loss of body weight that occurs with advancing, untreated HIV infection, PHPT may be caused by increased body weight, and vitamin D insufficiency is more common in people with increased body weight. Because of its central role in pathogenesis, it is therefore essential to consider body weight in any study of these causes of secondary osteoporosis.

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