VITAMIN D STATUS AND CARDIOMETABOLIC RISK FACTORS IN BLACK AFRICAN AND INDIAN POPULATIONS OF SOUTH AFRICA

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A thesis submitted to the Department of Chemical Pathology, Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I, Jaya Anna George hereby declare that this thesis is my work. It is being submitted for the degree of Doctor of Philosophy in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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Signed

Date : _____

DEDICATION

This thesis is dedicated to my husband Christopher Thomas for his love and unwavering support and
to my children Sunila and Benjamin for their enthusiasm, patience and encouragement.

AUTHORS CONTRIBUTION TO THE WORK

I was involved in all stages of the study. The original hypothesis was mine. I obtained informed consent, collected blood when the phlebotomist could not, administered sunshine exposure questionnaire and some food frequency questionnaires, and observed the process of DXA scanning and ultrasonography. I reviewed DXA scans for quality and artifacts, and reviewed all HPLC chromatograms for vitamin D. I calculated the weekly nutrient intake from the FFQs and then entered the data into the software to output the daily intake of calcium and vitamin D.

I carried out all the statistical analysis.

PUBLICATIONS ARISING FROM THIS THESIS

1.George JA, Norris SA, van Deventer HE, Crowther NJ. 2013 The association of 25 hydroxyvitamin D and parathyroid hormone with metabolic syndrome in two ethnic groups in South Africa. PLoS ONE 8:e61282 (appendix 6)

Contributions to the paper by each author:

Jaya A George: Conceptualization of the paper, data management, data analysis and primary write up.

Shane A Norris: Supervision of conceptualization of the study components and advisory role in write up.

Emmanuel van Deventer: Advisory role with statistical analysis

Nigel J Crowther: Supervision of conceptualization of the study, advisory role with statistical analysis and advisory role in write up.

2.George JA, Micklesfield LK, Norris SA, Crowther NJ. The association between body composition, 25(OH)D and PTH, and bone mineral density in Black African and Asian Indian population groups

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Jaya A George: Conceptualization of the paper, data management, data analysis and primary write up.

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Nigel J Crowther: Supervision of conceptualization of the study, advisory role with statistical analysis and advisory role in write up.

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CONFERENCE PROCEEDINGS

1. George JA, Norris SA, van Deventer HE, Crowther NJ.
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ABSTRACT

Background: South Africa is in the midst of a health transition that is characterized by a high burden of both infectious diseases and non-communicable diseases. One of the drivers of non-communicable diseases in South Africa is the current epidemic of obesity. Vitamin D deficiency, which is defined by 25(OH)D levels in blood, has been reported to be a risk factor for cardiovascular disease and shares a number of risk factors with those traditionally linked to non-communicable diseases. Osteoporosis is another non-communicable disease that is reportedly increasing in prevalence worldwide and may be linked to vitamin D levels and to body fat. There is limited data on 25(OH)D levels in South Africa and its association with cardiovascular risk factors. There is also limited data on body composition including bone mineral density.

Aims: The aims of this thesis were to describe 25(OH)D levels in healthy Black African and Indian subjects recruited from the greater Johannesburg metropolis and to determine if differences in 25(OH)D levels contributed to differences in cardiovascular risk. A further aim was to describe body composition in both ethnic groups and to see if differences in body composition contribute to differences in 25(OH)D levels or to differences in bone mineral density and to determine if differences in bone mineral density are mediated by differences in 25(OH)D.

Methods: This was a cross sectional study carried out from July 2011 to March 2012 on 714 male and female subjects (male: female=340:374) of whom 371 were Black African and 343 were Indian. Subjects were recruited via the caregivers of the Birth to Twenty cohort. The first step was a descriptive analysis of 25(OH)D as well as its predictors including whole body fat, visceral and subcutaneous adiposity. This was followed by examining the associations of 25(OH)D and parathyroid hormone with cardiovascular risk factors that comprise the metabolic syndrome. Final analysis was description of bone mineral density according to ethnicity and gender and the contribution of lean mass, sub-total fat mass, visceral and subcutaneous adiposity to bone mineral density in each ethnic group.

Results: Vitamin D deficiency was very prevalent in Indians, 28.6% in comparison to 5.1% in the Black African group ($p<0.0001$). In both groups season of collection was a positive predictor and PTH was negatively associated with 25(OH)D. Neither whole body fat nor visceral or subcutaneous adiposity was predictive of 25(OH)D in either group. Using the harmonized definition of the metabolic syndrome (Met S), was diagnosed in 29% of the Black African and 46% of the Indian subjects ($p<0.0001$). Subjects with Met S had higher PTH than those without ($p<0.0001$), whilst 25(OH)D levels were not significantly different ($p=0.50$). Logistic regression analysis showed that Indian ethnicity

(OR 2.24; 95% CIs 1.57, 3.18; p<0.0001) and raised PTH (OR 2.48; 95% CIs 1.01, 6.08; p=0.04) adjusted for 25(OH)D) produced an increased risk of Met S but 25(OH)D did not (OR 1.25; 95% CIs 0.67, 2.24; p=0.48). Whole body, hip, femoral neck and lumbar spine bone mineral density were significantly higher in Black African than Indian subjects (p<0.001 for all). Whole body lean mass positively associated with bone mineral density at all sites in both ethnic groups (p<0.001 for all), and partially explained the higher bone mineral density in Black African females compared to Indian females. Whole body fat mass correlated positively with lumbar bone mineral density in Black African (p=0.001) and inversely with sub-total bone mineral density in Indian subjects (p<0.0001). Visceral adiposity correlated inversely with sub-total bone mineral density in the Black African subjects (p=0.037) and with lumbar bone mineral density in the Indian group (p=0.005). No association was found between serum 25(OH)D and bone mineral density. PTH was inversely associated with hip bone mineral density in the Black African group (p=0.01) and with sub-total (p=0.002), hip (p=0.001) and femoral bone mineral density (p<0.0001) in the Indian group.

Conclusions: This study highlighted the high prevalence of vitamin D deficiency in the Indian population and the fact that local conditions such as sunshine exposure and season of collection of blood are important determinants of 25(OH)D levels. It also showed that Indian ethnicity and PTH are risk factors for the Met S, but differences in risk between both ethnic groups are not due to differences in 25(OH)D levels. The thesis also showed that there are significant differences in bone mineral density across ethnicity, with lean mass an important contributor to bone mineral density across race and gender.

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LIST OF ABBREVIATIONS

BMD	:	Bone mineral density
BMI	:	Body mass index
CAD	:	Coronary artery disease
CVD	:	Cardiovascular disease
Cohort	:	Defined by the INDEPTH Network (2002) as a “group of people sharing a common temporal demographic experience who are observed through time”.
CV	:	coefficient of variation
DALYS	:	Disability-adjusted life years
DBP	:	Vitamin D binding protein
DEQAS	:	Vitamin D External Quality Assessment Scheme
DXA	:	Dual-energy X-ray Absorptiometry
eGFR	:	Estimated glomerular filtration rate
FGF23	:	Fibroblast growth factor 23
HbA1c	:	Glycated haemoglobin
HIV	:	Human Immunodeficiency Virus
HOMA	:	Homeostatic model of assessment
HPLC	:	High performance liquid chromatography
Indian	:	Refers to people of Indian sub-continent extraction
Africans	:	Refers to black South Africans
IOM	:	Institute of Medicine
IR	:	Insulin resistance
LC/MS	:	Liquid chromatography mass spectrometry
Met S	:	Metabolic Syndrome
NADP	:	Nicotinamide adenine dinucleotide phosphate
NCD	:	Non-communicable diseases
NHANES	:	National Health and Nutrition Examination Survey
NIST	:	National Institute of Standards
OGTT	:	Oral glucose tolerance test
PTH	:	Parathyroid hormone
RIA	:	Radioimmunoassay
SCAT	:	Subcutaneous tissue
25(OH)D	:	25 hydroxy-vitamin D
$1,25(OH)_2D$	:	1α, 25 –hydroxyvitamin D
T2DM	:	Type 2 Diabetes Mellitus

US	:	Ultrasound
UVA	:	Ultraviolet A
UVB	:	Ultraviolet B
VAT	:	Visceral adipose tissue
VDR	:	Vitamin D receptor
WHO	:	World Health Organization

PREFACE

The idea for this study came as a result of the introduction of a high performance liquid chromatography (HPLC) method, in our laboratory, for the measurement of 25-hydroxy vitamin D (25(OH)D) which is the main metabolite of vitamin D . Volunteers were required for a method comparison study. Three Indian pathologists including myself donated five ml of blood for the comparison study and all of us were noted to be vitamin D deficient.

Obesity has reached global epidemic proportions in both adults and children and is associated with several comorbidities including type 2 diabetes mellitus (T2DM), hypertension, dyslipidaemia and major cardiovascular disease. Inverse relationships have been demonstrated between circulating levels of 25(OH)D and obesity as well as with the metabolic syndrome (Met S) or the metabolic abnormalities associated with it, namely, elevated fasting glucose and insulin resistance (IR)/diabetes. Osteoporosis also of major public health importance and it shares a number of risk factors with cardiovascular disease namely lack of exercise, smoking and possibly low vitamin D status. While there is some data on the prevalence of the metabolic syndrome in various populations here, there is very little data on vitamin D status of the various ethnic groups in South Africa. I was intrigued to investigate if there were differences in vitamin D status of local populations living in Johannesburg, an area that receives at least eight hours of sunshine per day throughout the year, and if these differences contributed to differences in cardiometabolic risk factors.

I was fortunate to have access to the caregivers of the Birth to Twenty cohort which allowed me to recruit healthy male and female adults from two population groups. The infrastructure at Birth to Twenty also allowed me to perform Dual-energy X-ray Absorptiometry scans, ultrasound measurements of adiposity and dietary intake of all subjects assessed by a seven day food frequency questionnaire. Thus the findings of this thesis fill in a gap not only on knowledge of the relationship between 25(OH)D and risk of the Met S but also on body composition of African men in particular.

This PhD thesis is presented as 6 chapters:

- Chapters 1 and 2 are the literature review and methods chapters, respectively.
- Chapters 3 to 5 contain the results of the studies undertaken for this PhD, with each chapter written in the format of a research publication. Chapter 4 has been published as a paper, whilst the other 2 chapters have been submitted and are currently under review by the respective journals.
- Chapter 6 comprises of the conclusions and recommendations for further work.

CHAPTER 1

1. LITERATURE REVIEW

1.1 INTRODUCTION

Up to the middle of the previous century, infectious diseases were the main causes of death worldwide. Then with strides in medicine such as vaccinations and antibiotics as well as with improved socio-economic conditions, non-communicable diseases (NCD) became increasingly important as a cause of morbidity and mortality in the industrialised world. The dawn of the third millennium saw NCDs becoming common throughout the world with an increasing trend in developing countries. The leading causes of disease burden in 1990 were pneumonia, diarrhoeal diseases and complications of pregnancy and childbirth but by 2020 it is estimated that NCDs will account for 80 percent of the global burden of disease, causing seven out of ten deaths (1, 2). A World Health Organization report of 2011 noted that ischaemic heart disease was responsible for seven million deaths worldwide (Figure 1.1) (3).

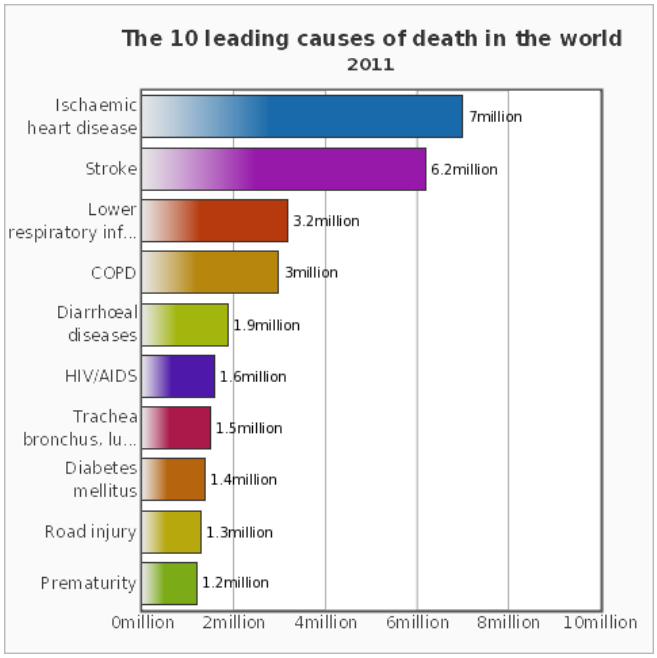

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Figure 1.1: **The 10 Leading Causes of Death in the World** (*taken from reference number(3)*)

Deaths from NCD account for three out of every ten deaths worldwide and this is set to increase (4). The International Diabetes Federation (IDF) estimates that the number of people living with diabetes will increases from 366 million in 2011 to 552 million by 2030 (5). Eighty percent of these will be from low and middle-income

countries. The projected increase will contribute to increased mortality as well as disability adjusted life years (DALY) and sum of years lived with disability (YLD). In developing countries, those most frequently affected are between the ages of 35 and 64. Although the numbers of premature deaths from diabetes are similar to that of HIV/AIDS, the problem is largely unrecognized.

South Africa, a low to middle-income country (LMIC), is in the midst of a health transition characterized by the simultaneous occurrence of epidemic infectious diseases, a rise in NCDs such as diabetes, heart disease, chronic pulmonary and mental diseases. The World Health Organization (WHO) estimates that the burden of disease in South Africa arising from NCDs is two to three times higher than in developed countries, and that NCDs are increasing in prevalence and disproportionately affect poor people living in urban areas (6). There are several reasons for this rise including an increased number of people over the age of 60, and risk behaviour such as smoking and lifestyle factors which lead to obesity (7). As illustrated in Figure 1.2, from 1999 to 2006 there have been sustained increases in diabetes and hypertensive heart disease in South Africa (Figure 1.2).

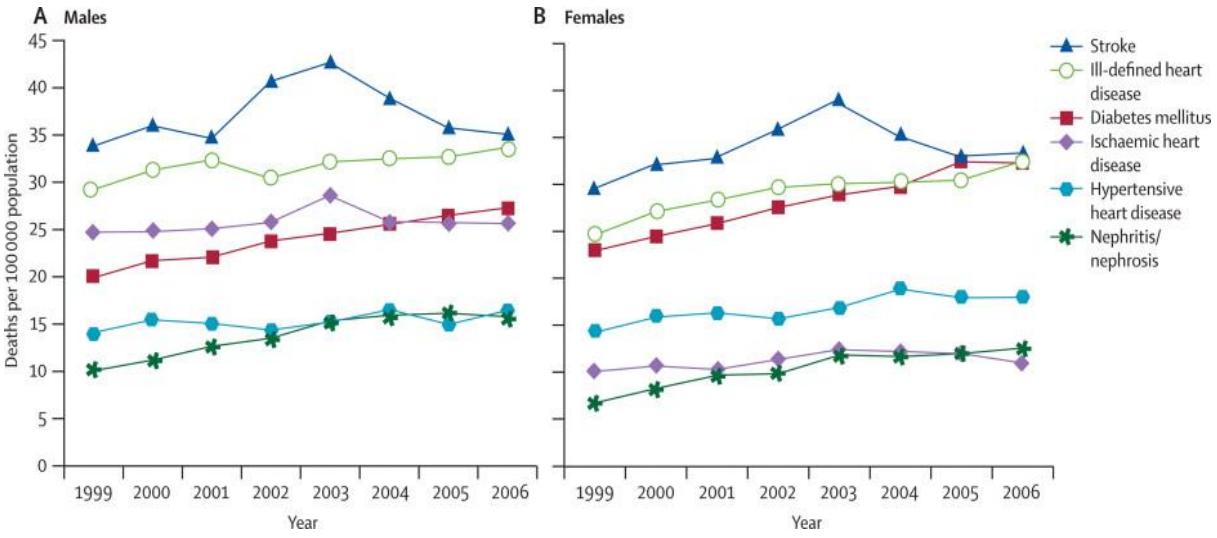


Figure 1.2: **The Burden of Non-Communicable Diseases in South Africa from 1999-2006** (*taken from reference (6) and used with permission*)

Interrelationships between risk behaviour, risk factors and disease outcome are illustrated in Figure 1.3.

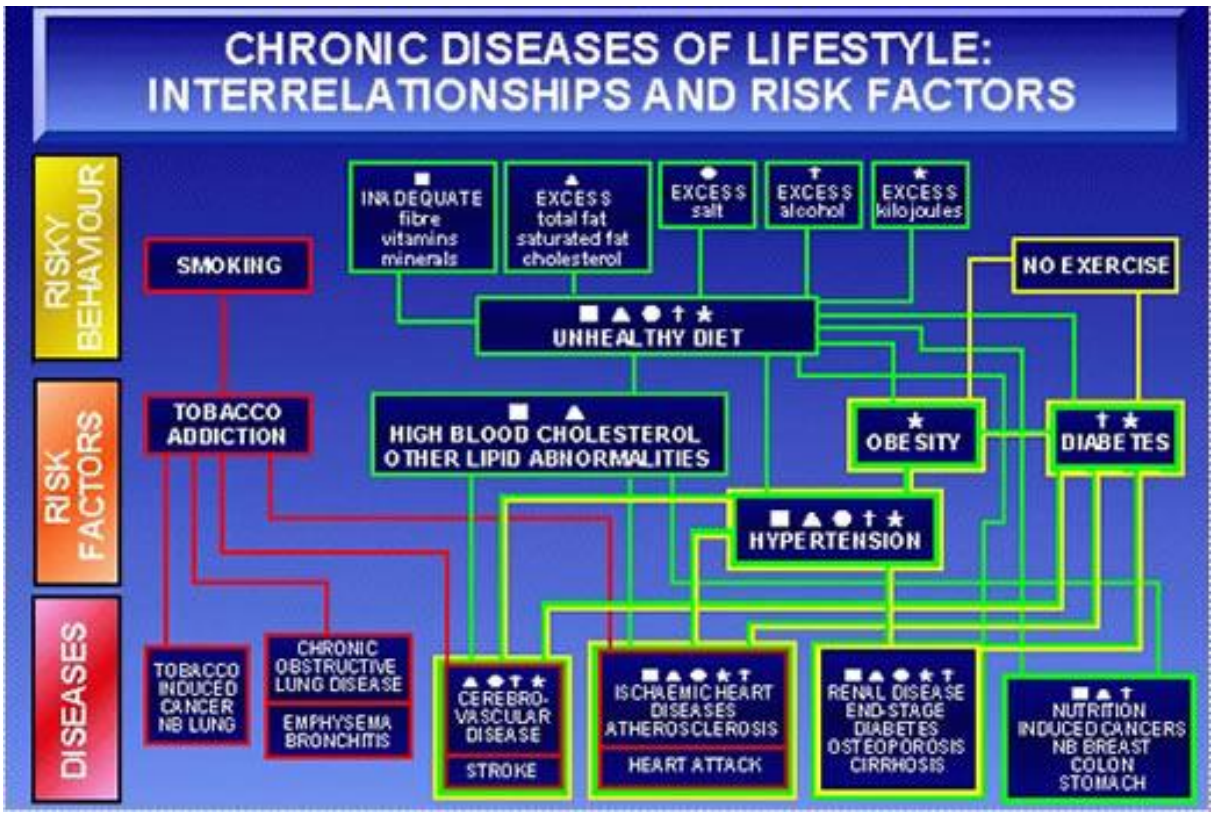


Figure 1.3: **Interrelationships between Risk Factors and Non-Communicable Diseases** (*taken from reference (8) and used with permission*)

Obesity, previously thought to be a disease of affluence, has reached epidemic proportions with prevalence rates of up to 30% in African women (9). Overall, it appears that overweight and obesity may already be more prevalent than underweight and under nutrition in many developing countries (10). Vitamin D deficiency shares several risk factors and risk behaviours with those traditionally linked to NCDs particularly cardiovascular disease (Figure 1.4) and has become pandemic (11-13).

Both cardiovascular disease and vitamin D deficiency are strongly linked to obesity.

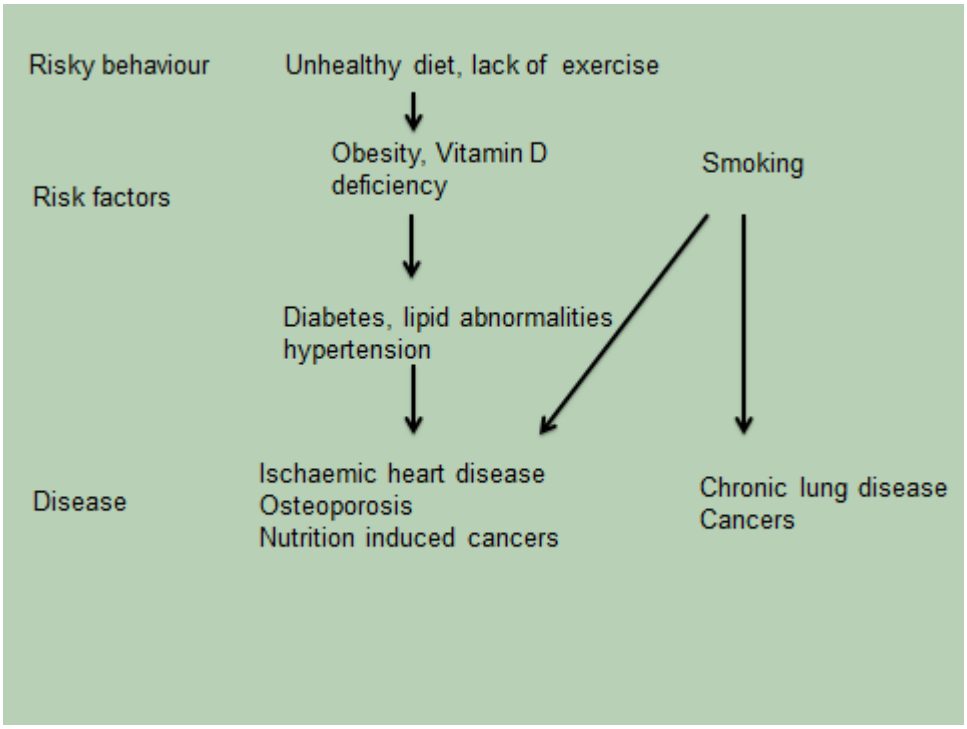


Figure 1.4: Possible Interrelationships between Vitamin D Deficiency, Traditional Risk Factors and Non-Communicable Diseases

There is a lack of data on the vitamin D status of South African populations and any possible association of vitamin D with cardiovascular risk factors. It is important that we understand the drivers of NCDs and define the role played by non-traditional risk factors like vitamin D deficiency in the aetiology of obesity-related disorders.

The next section gives an overview on the definition and epidemiology of the metabolic syndrome (section 1.2), whilst Section 1.3 covers the history, physiology and the measurement of vitamin D. Section 1.4 includes information on the prevalence of vitamin D deficiency, and section 1.5 is a review of studies on the association of vitamin D with components of the metabolic syndrome.

1.2 THE METABOLIC SYNDROME

The prevalence of obesity is reportedly quite different among the four major South African population groups. Thus, it is highest in African women at 31-34%, 20-22% in Indian women, 8% in African men and 3-9% in Indian men (9). The drivers of this obesity epidemic include rural to urban migration, sedentary lifestyle and adoption of high calorie western diets (14). The particularly high prevalence of

obesity seen in Black African women is similar to that seen in African-American women in the United States (6, 15). In addition to total body fat, the pattern of body fat distribution is as an important risk factor for dysglycaemia, hyperlipidaemia and hypertension in patients with and without T2DM. Factors involved in the control of body fat distribution include gender, age and ethnicity. Women have more total body fat than men even when matched for BMI (16), because women have a larger subcutaneous adipose tissue depot than men, but less visceral fat (17). This gender difference is apparent prenatally and from the first year of life (18). Abdominal obesity, represented by waist circumference or waist-hip ratio is considered particularly atherogenic because it causes dysregulation of a number of metabolic processes including those controlling glucose, lipids and blood pressure. Thus begins a clustering of metabolic and cardiovascular conditions: dysglycaemia, dyslipidaemia, hypertension and a procoagulant state which are collectively known as the metabolic syndrome (Met S) (6, 19).

The first formalized definition of the Met S was proposed in 1998 (20). This definition emphasized insulin resistance as the major risk factor and required evidence of insulin resistance for diagnosis. Since then there have been several definitions using different criteria (21-23), leading to widely different prevalence estimates (22). The two major sets of criteria that have been used are those of the National Cholesterol Education Program Third Adult Treatment Panel (ATP III) (21, 24, 25) and the International Diabetes Federation (IDF) (23). The ATP criteria placed more emphasis on cardiovascular risk and required three of any five components: central obesity, raised blood pressure, raised triglycerides, low HDL cholesterol and fasting hyperglycaemia. The IDF placed central obesity as essential to the diagnosis of the metabolic syndrome and suggested ethnic specific cut off points for waist circumference. In 2009, an additional definition was proposed, as a joint interim statement (JIS) by several organizations. This was an attempt to harmonize the definition of the Met S (22). They determined that there should not be an obligatory component, three out of five components would be used and ethnic specific cut off points be used for waist circumference. The available information based on the ATP III and the IDF criteria suggests that the Met S is pandemic but that prevalence varies widely depending on the ethnic groups studied and the criteria applied (26).

The Met S can be explained by viewing abdominal adipose tissue as an endocrine organ that secretes a number of bioactive compounds into the circulation. Many of

these molecules have inflammatory effects (adipokines) but also modulate insulin sensitivity, blood pressure, lipid levels and a number of other cardiovascular and metabolic pathways, all of which are involved in the aetiology of obesity-related diseases (27).

With the rising prevalence of obesity, the Met S is also increasing in developing countries. The prevalence of the Met S is high in Black Africans from urban and rural parts of South Africa. (28, 29). A cross sectional diabetes epidemiological study in rural South Africans found the prevalence to be 26.5% using the harmonized definition (28) and in in urban Black Africans with coronary artery disease (CAD) it was shown to be much higher at 60% (29). Indians have an unusually high tendency to develop T2DM and CV (30). There are no data on the prevalence of the Met S in Indian living in South Africa, however data from India indicate a prevalence of between 11-41% depending on the area and the criteria used to define it (31).

The Met S gives a 2 to 3 fold increased risk for coronary artery disease (CAD), a similar risk for ischaemic stroke (32) and a much greater risk for future diabetes (33, 34). The more features of the Met S an individual has, the greater the risk of metabolic disease (35).

It has been suggested that the Met S may be a risk factor for osteoporosis as they share a number of common risk factors including a sedentary lifestyle, smoking, sex-hormone deficiency and oxidative stress (36-38). Increased body weight is inversely associated with bone mineral density, however studies analysing the association of abdominal obesity with BMD have contradictory findings (39). Studies from various parts of the world suggest that vitamin D deficiency may increase the risk of the Met S (40, 41) and that vitamin D repletion might prove protective against Met S and its sequelae, T2DM and cardiovascular disease (42). In addition, vitamin D deficiency leads to secondary hyperparathyroidism that can precipitate and exacerbate osteopaenia and osteoporosis in adults (43).

There is limited data from Africa on the prevalence of vitamin D deficiency and its possible association with components of the Met S or BMD.

1.3 THE DISCOVERY OF VITAMIN D

The discovery of vitamin D (44) followed by the virtual elimination of rickets ranks as one of the greatest and most fascinating medical achievements. In the nineteenth century, with the onset of the industrial revolution, people in Europe moved from rural farming areas to smoggy urban areas and rickets became widespread. Sir Edward Mellanby reasoned that rickets might be caused by a nutritional deficiency. He reasoned that he could produce rickets in dogs by feeding them oatmeal, the staple diet of the Scottish. What he did not realise was that he also deprived the dogs of sunlight. He then demonstrated that these dogs were cured of their rickets when fed cod liver oil (45) and attributed this effect to vitamin A. Earlier on, vitamin A had been discovered by McCollum and Davis, who showed that it prevented xerophthalmia (46). When McCollum heated cod liver oil, which destroyed vitamin A, he showed that the cod liver oil still cured rickets. He concluded that there was another vitamin in cod liver oil with anti-rachitic properties and as vitamins A, B and C had already been discovered he named it vitamin D (47). At the same time that Mellanby was experimenting on dogs, Huldshinsky and Chick working independently, showed that rickets in children could be cured either by exposing them to sunlight or artificially produced ultraviolet light (48). Steenbock and Hart, who were working on the calcium balance of lactating goats, showed that irradiation of the animals or their diets could cure rickets. Steenbock traced this property to the nonsaponifiable fraction of fat in food. He found that ultra violet (UV) light activated a substance that then showed anti-rachitic properties. This discovery, led to the widespread use of irradiation of food to increase the vitamin D content, and culminated in the virtual elimination of rickets. A British group led by Askew determined the structure of vitamin D_2. Windaus and Bock confirmed the structure of vitamin D_2 and furthermore isolated 7-dehydrocholesterol, the precursor of vitamin D_3 and then produced D_3 from its precursor (48).

1.3.1 Physiology of Vitamin D

Terminology

Various molecules may be non-specifically referred to as vitamin D and it is important to define the relationships and functions of these molecules. The nomenclature is derived from the historical sequence of identification of substances found to have anti-rachitic activity. Vitamin D_3 is a prohormone produced by ultraviolet irradiation (UVB) of 7-dehydrocholesterol in skin (Figure 1.1). Due to individual variability in sun exposure there are considerable

differences in the amounts of vitamin D produced by this route (49). Diet and dietary supplements are also sources that provide either vitamin D_2 (ergocalciferol) or D_3 (cholecalciferol). Either way, they must be activated by metabolism to 25-hydroxyvitamin D [25(OH)D] in the liver and then to 1α,25 hydroxyvitamin D in the kidney [1,25$(OH)_2$D]. 1,25$(OH)_2$D acting via its nuclear receptor carries out both skeletal and extra skeletal functions. Ultimately, vitamin D metabolites are excreted by further hydroxylation to 24,25$(OH)_2$D, which increase their water solubility and the metabolites are excreted in bile.

Vitamin D Binding Protein

The generation of vitamin D_3 occurs in the plasma membrane from where it then moves to the extracellular fluid and is bound to vitamin D binding protein (DBP) (50). Vitamin D binding protein, a member of the albumin and alpha-fetoprotein gene family, is a serum transport protein for all vitamin D metabolites. It is synthesized in the liver and serum concentrations range between 4-8µmol/L (51, 52) and has a half-life of 30 to 72 hours. Levels are raised in pregnancy and are lowered in liver disease, nephrotic syndrome and malnutrition but are not affected by levels of 25(OH)D or any of the metabolites (53). DBP serves as a reservoir for circulating 25(OH)D and 1, 25$(OH)_2$D. In addition it binds several other proteins and is crucial in the clearing of actin filaments from the microcirculation (54).

Vitamin D Hydroxylases

Both vitamins D_2 and D_3 have no known biological function and are active only after conversion to 1,25$(OH)_2$D in the kidney. This occurs via a series of reactions: the first of which is a hydroxylation step in the liver. There are two enzymes in the liver capable of hydroxylating vitamin D, a microsomal hydroxylase (CYP2R1) and a mitochondrial hydroxylase (CYP27A1).

CYP2R1 is primarily expressed in the liver and the testis (55) and is believed to be the main enzyme involved in hydroxylation. Genetic loss of CYP2R1 leads to deficient 25-hydroxylation of D_3 demonstrating its physiological importance (56). Recent genome wide association studies (57) have implicated CYP2R1 as one of the four major determinants of 25(OH)D, the others being DBP, CYP24A1 and 7-dehydrocholesterol reductase (DHR7). Further evidence that strengthens the case for CYP2R1 being the functional 25 hydroxylase is the presence of a mutation Leu99Pro, which results in vitamin D dependent rickets type 1 (58). Biological and clinical evidence argue against CYP27A1 being a physiologically relevant 25-

hydroxylase. Firstly, the null mouse phenotype does not include any bone lesions (59). Secondly, human mutations result in a bile-acid related disorder known as cerebrotendinous xanthomatosis rather than rickets (60) and finally, the genome wide association study of the determinants of serum 25(OH)D concentrations concluded that variants at the locus for CYP2R1 but not CYP27A1 are associated with serum 25(OH)D concentrations (57). CYP2R1 does not appear to be subject to feedback regulation. 25(OH)D is biologically inactive and must be converted by CYP27B1 to the biologically active form $1,25(OH)_2D$. This occurs in the kidney as well as in extra renal tissue such as placenta, skin, colon, macrophages, prostate and breast (61, 62). Extra renal CYP27 B1 is not subject to regulation by $1,25(OH)_2D$, PTH, calcium or phosphate while the main influences on $1,25(OH)_2D$ production in the kidney are by $1,25(OH)_2D$ itself, parathyroid hormone as a signal of calcium status, and fibroblast growth factor 23 (FGF23) as a signal of phosphate homeostasis.

25(OH)D and $1,25(OH)_2D$ are inactivated by CYP24A1. This multi-catalytic enzyme inactivates 25(OH)D to 24,25(OH)D and other metabolites. It also converts $1,25(OH)_2D$ to water-soluble calcitroic acid.

Mode of Action of $1,25(OH)_2D$

$1,25(OH)_2D$, like many other steroid hormones mediates its actions by the regulation of the expression of specific genes and by rapidly activating signal transduction pathways. Genomic responses to $1,25(OH)_2D$ result from its interaction with the vitamin D nuclear receptor (VDR) (63). This receptor does not bind vitamin D_3, binds 25(OH)D weakly and binds $1,25(OH)_2D$ with high affinity. It is similar to other nuclear receptors in that it has six functional domains: the variable regions (A and B domains), the DNA binding domain (C domain), the hinge region (D domain), the ligand binding region (E domain) and the transcriptional activation domain i.e. the F domain. Binding of $1,25(OH)_2D$ to its nuclear receptor causes a tight association of VDR with its heterodimeric partner the retinoic X receptor (RXR) (64). The VDR-RXR complex is able to recognize vitamin D response elements in vitamin D regulated genes. $1,25(OH)_2D$ also binds to VDR in caveolae of plasma membranes and generates rapid non-genomic responses via activation of second messenger systems (65). $1,25(OH)_2D$ down regulates its own production (66) and it is thought that this is mediated by interaction of the steroid hormone with its nuclear receptor.

Macrophages and dendritic cells express VDR, which suggests that vitamin D plays an important role in modulating the immune response. In both macrophages and dendritic cells CYP27B1 is regulated by cytokines e.g. interferon -γ (IFN-γ) and other inflammatory mediators such as lipopolysaccharides (LPS) (67). One of the most important cytokines released by macrophages is tumour necrosis factor alpha (TNF-α) (68). $1,25(OH)_2D$ has been shown to inhibit the expression of a number of pro-inflammatory cytokines including C-reactive protein, interleukin-1β, interleukin-6 and TNF-α and up regulate the anti-inflammatory cytokine, interleukin-10 (69). These actions therefore have the potential to dampen the pro-inflammatory cascade associated with CVD.

The clinical evidence demonstrating the importance of the VDR for normal calcium absorption is from patients with type II vitamin D dependent rickets who have an inactivating mutation in the VDR gene (70). Laboratory evidence is from VDR knockout mice where deletion of the receptor leads to a 70% or greater reduction in calcium absorption as well as poor growth, low serum calcium and high parathyroid hormone (PTH) along with osteomalacia (71).

$1,25(OH)_2D$ Regulation of Serum Calcium

$1,25(OH)_2D$ acts directly to increase calcium absorption from the gut (Figure 1.5) via multiple mechanisms including facilitated diffusion, transport mechanisms utilizing calbindin and passive transport mechanisms (72).

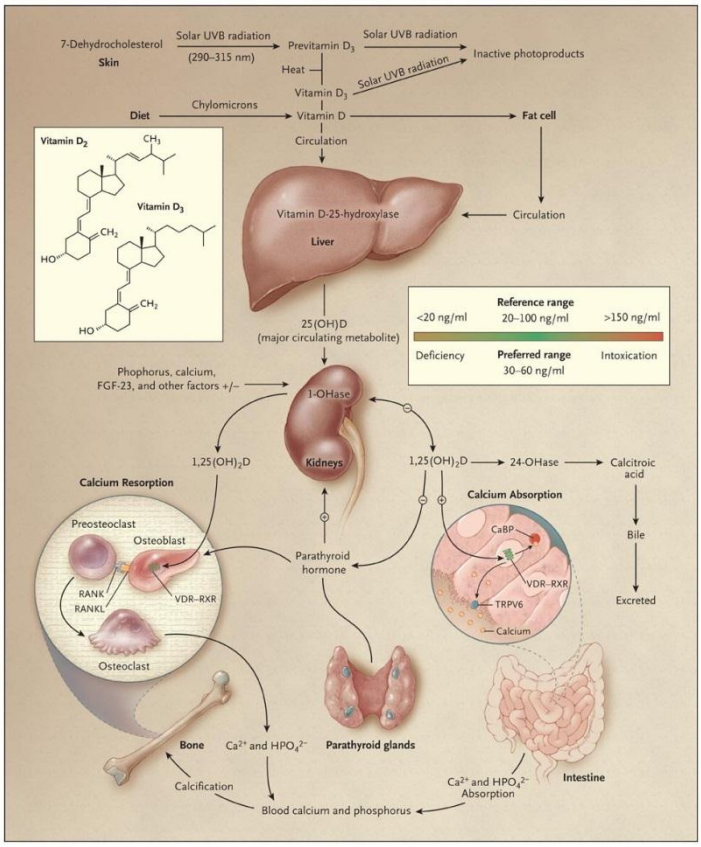


Figure 1.5: Synthesis and Metabolism of Vitamin D in the Regulation of calcium, Phosphorus and Bone Metabolism (*taken from reference (73) and used with permission*)

Additionally, 1,25$(OH)_2$D regulates serum calcium via its effects on PTH. PTH is involved in maintaining normal serum levels of calcium and phosphate and is itself regulated through 1,25$(OH)_2$D and serum calcium. PTH activates CYP27B1 in the proximal tubule and thus increases 1,25$(OH)_2$D. Vitamin D receptor mRNA has been localized to the parathyroid glands and cell culture studies have shown that the addition of 1,25$(OH)_2$D led to a marked decrease in PTH mRNA, followed by a decrease in PTH secretion (74). In addition to its direct effects on PTH mRNA expression, 1,25$(OH)_2$D may also act on PTH secretion by modulating the activity of the calcium sensing receptor (CaSR). This is a G-protein coupled receptor (GPCR) predominantly expressed in kidney and parathyroid glands where it allows regulation of PTH secretion and renal tubular calcium absorption in response to the prevailing calcium concentration. High plasma Ca^{2+} concentrations suppress PTH secretion, gene expression, PTH cellular proliferation and PTH degradation (75). The CaSR is also expressed in other tissues including the thyroid, intestine, bone, brain, heart and lung where its function is unknown (76). Parathyroid

hormone acts via a number of organs to regulate serum calcium. This is summarised in Table 1.1 below.

Table 1.1: Effects of Parathyroid Hormone in the Regulation of Serum Calcium

Organ	Effect
Kidney: distal tubule	Increased tubular reabsorption of calcium[50]
Proximal tubule	Increased phosphate excretion due to decreased phosphate transporter protein[77]
Proximal tubule	Activates CYP27B1 and thus increases $1,25(OH)_2D$[50]
Bone	Osteoblasts; increased proliferation, differentiation, activity[78]
	Up regulates Receptor Activator of Nuclear Factor κ β ligand (RANKL) and inhibits osteoprotegerin (OPG) and thus increases osteoclastogenesis[79]

The decrease in PTH gene transcription effected by $1,25(OH)_2D$ is used in the management of patients with chronic kidney disease (CKD). Such patients are treated with $1,25(OH)_2D$ or a prodrug to reduce the secondary hyperparathyroidism seen in CKD.

Phosphate regulates PTH gene expression indirectly by reducing calcium levels, via Fibroblast Growth Factor 23 (FGF23) which suppresses the production of $1,25(OH)_2D$, and by direct effects on the parathyroid gland. FGF-23 is a circulating hormone produced by osteocytes, osteoblasts and endothelial cells. FGF-23 acts as a vitamin D counter regulatory hormone. It reduces $1,25(OH)_2D$ via down regulation of CYP27B1 and up regulation of CYP24 to decrease production of and increase catabolism of $1,25(OH)_2D$ (80). FGF-23 along with other factors promotes the urinary excretion of phosphate (81, 82). FGF23 signals through its receptors FGFRs bound by a transmembrane protein Klotho (83) and acts on the parathyroid gland to decrease serum PTH.

Both calcium and phosphate are filtered, reabsorbed and excreted by the kidney to varying degrees, generally in amounts that reflect the endogenous requirements of both substances. The kidney is the major site of production of $1,25(OH)_2D$ as discussed in section 1.3.1 and the vitamin D endocrine system plays an important role in controlling the renal excretion of both calcium and phosphorus. The kidney expresses the enzymes involved in metabolism of $1,25(OH)_2D$, as well as the VDR

and several of the proteins involved in calcium transport, including the plasma membrane calcium pump (84, 85), the epithelial calcium channel (86), the sodium calcium exchanger (87) and calbindin (84).

1.3.2 Dietary Sources of Vitamin D

The main dietary sources are summarized in Table 1.2

Table 1.2: Dietary and Supplemental Sources of Vitamin D_2 and Vitamin D_3

Source	Vitamin D Content (approximate)
Fish: Salmon Fresh, wild Fresh, farmed Canned Sardines, canned Mackerel, canned Tuna, canned	 600-1000IU of vitamin D_3 100-250IU of vitamin D_2 or D_3 300 to 600 IU of vitamin D_3 300IU of vitamin D_3 250 IU of vitamin D_3 230IU of vitamin D_3
Cod liver oil	400 to 1000IU of vitamin D_3
Egg yolk	20 IU of vitamin D_2 or D_3
Cereals	Variable
Supplements: Ergocalciferol Vitamin D3 Multivitamin	 50 000IU supplements 400, 800, 1000 and 2000IU 400 IU vitamin D, D_2 or D_3

Modified from(73)

1.3.3 Cutaneous Production of 25(OH)D

The amount of 25(OH)D produced in the skin is dependent on the amount of substrate, namely 7-dehydrocholesterol, which is affected by increasing age (88) and also by exposure of the skin to UVB, which is in turn affected by geographical, physical and cultural conditions. Geographical conditions that influence 25(OH)D status include season (89) as well as time of day. Some of these studies are summarized in Table 1.3 below.

Table 1.3: Seasonal Variation in 25(OH)D Levels

Location	Population	Winter 25(OH)D nmol/L	Summer 25(OH)D nmol/L	Reference
United States, Massachusetts	Healthy subjects , male and female	62	77	(90)
New South Wales Australia	Males and females retrospective patient samples	47	63	(91)
Cape Town South Africa	Healthy, HIV positive and negative with and without TB	31	57	(92)[a]
Brazil	Students and staff at university	55	85	(93)

a) Winter to early spring and late summer to autumn

It was thought that distance from the equator modified 25(OH)D levels. Theoretically regions closer to the equator should have higher levels of 25(OH)D, due to longer periods of sunlight and this appeared to be confirmed by studies from North America years ago (89, 94). However this has been questioned after Hagenau et al conducted a meta-analysis of cross–sectional studies on serum 25(OH)D globally and suggested that after adjusting for age and gender there was no trend of changing 25(OH)D levels with latitude (95). Observed differences were attributed to differences in lifestyle, supplementation, fortification policy etc. The data on seasonal variation are more convincing, with studies from across the globe reporting a seasonal variation in 25(OH)D levels for all age groups. While levels are often lower in winter (89, 91, 92, 96) in some very hot countries 25(OH)D levels have been shown to be lower in summer as people avoid the sun by staying indoors (97). Individual factors that impair 25(OH)D production include dark skin which requires longer exposure to sunlight to produce 25(OH)D in equal amounts as light skin (98), cultural practices such as veiled clothing (99) and possibly the use of sunscreens. These are used to protect the skin from ultraviolet A and B (UVA and UVB) exposure; the same UVB exposure is needed for vitamin D synthesis. Experimental studies suggest that the use of sunscreens can decrease vitamin synthesis in the skin (100) however recent studies suggest that while sunscreens are effective at blocking UVB, they are often applied improperly and therefore may not actually diminish vitamin D synthesis (101). Limiting outdoor activities can further reduce the amount of sun exposure and thus cutaneous exposure. The elderly, hospitalised or institutionalised individuals are at increased risk of lower 25(OH)D levels compared to healthy adults (102, 103). Toxicity does not occur with prolonged exposure to sunlight because $25(OH)D_3$ can also undergo thermal isomerisation to inactive products, such as lumisterol and tachysterol.

1.4 OTHER FACTORS THAT AFFECT VITAMIN D SUPPLY AND METABOLISM

Parathyroid Hormone

Parathyroid hormone reduces the half-life of 25(OH)D and anything that causes secondary hyperparathyroidism may result in depletion of 25(OH)D levels. Cases of calcium deficiency induced rickets have been reported from several countries (104-106). The mechanism is via the resulting elevation in PTH and $1,25(OH)_2D$ levels and a decrease in 25(OH)D half-life (107).

Renal Function

As illustrated in Figure 1.1, 25(OH)D is converted to its active metabolite in the kidney. Poor renal function acts in number of ways to reduce 25(OH)D metabolism. For example, impaired renal function may reduce the conversion of 25(OH)D to $1,25(OH)_2D$. In addition, the resulting secondary hyperparathyroidism reduces the half-life of 25(OH)D and thus its concentration in circulation (108).

Cultural Practices

Cultural practices such as clothing are important contributors to differences in 25(OH)D concentrations. The religious practice of the veil, which often involves total covering of the body except for the eyes, is an independent risk factor for hypovitaminosis D in the Middle East and Africa (109, 110). Dietary practices also affect 25(OH)D concentrations. Studies from the polar regions of Canada have shown that consumption of fatty fish is associated with higher plasma levels of 25(OH)D (111). Ethnic differences in the consumption of dairy products, in countries such as the US, where fortification is policy, are reported among Asian and African immigrants and may be a contributing factor to differences in 25(OH)D levels (13).

1.5 GENETICS

The hereditability of vitamin D status appears to be considerable. A genome wide association study of people of European ancestry showed that variations in *GC* (which encodes the DBP), the *NADSYN1/DHCR7* locus and *CYP2R1* which encode enzymes in the vitamin D metabolic pathway are associated with circulating 25(OH)D levels (112). Similar findings have been noted by a number of other studies (113, 114) and have been replicated in African-Americans (115). In Gujurati-Asians, the Gc phenotype was shown to be associated with an increased prevalence of tuberculosis but not with lower 25(OH)D levels (116). It is important to note that these are associations, but direct cause has not been proven. However, if future studies identify significant associations between polymorphisms

and 25(OH)D levels, it will allow for potential identification of at risk individuals and early supplementation if required.

The effects of body fat mass and distribution on vitamin D levels will be discussed in detail in section 1.9.6.

1.6 MEASUREMENT OF 25(OH)D

The results of cross-sectional studies showing an association between vitamin D deficiency and diseases such as osteoporosis, T2DM and the Met S, have driven a rapid increase in 25(OH)D testing. Both immunoassays and chemical methods such as high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC/MS) are used by laboratories.

The first assays for the measurement of 25(OH)D were described in the 1970s and were based on competitive binding after solvent extraction. In 1985, the first radioimmunoassay (RIA) for the measurement of 25(OH)D was developed and this used a specific 25(OH)D antibody. Since then immunoassays employing either enzymes or chemiluminescent substrates have largely replaced RIAs. Later on, methods based on high performance liquid chromatography were developed. With the increasing use of tandem mass spectrometry for small molecule work, such methods are also increasingly being used.

25(OH)D is measured as a reflection of body stores of vitamin D because it has a longer half-life than vitamin D_2 and vitamin D_3 ($t_{1/2}$ of three weeks compared to 24 hours) and the 25 hydroxylase enzyme is not subject to significant regulation. Laboratory testing of 1,25$(OH)_2$D is also available but this is not used in the assessment of vitamin D deficiency as it has a $t_{1/2}$ of about four hours, concentrations are highly regulated and it is raised in cases of secondary hyperparathyroidism (117).

The very lipophilic nature of the compound, the high concentrations of DBP and the cross-reactivity of metabolites complicate analysis of 25 (OH) D. DBP binds with equal high affinity to 25(OH)D_2 and 25(OH)D_3 and less than 1% of the hormone is found in the free form. The US Food and Drug Administration require that assays measure both 25(OH)D_2 and 25(OH)D_3. Normally 25(OH)D_3 comprises 95% of total circulating 25(OH)D unless the patient is on supplements (118, 119)

Isotope dilution mass spectrometry is considered the gold standard for the measurement of small molecules, however, until recently there was no reference method available for 25(OH)D. More recently, two candidate reference methods were produced, one from the US and one from Europe in 2010 and 2011 respectively (120, 121). In addition and importantly, the first standard reference material was introduced in 2009 (SRM 972) and these measures are expected to improve the analytical performance of methods and allow for harmonization across platforms.

Chemical methods for the measurement of 25(OH)D include HPLC and LC/MS. Prior to chromatographic extraction an initial purification step is performed. Various deproteinization and extraction steps are performed prior to analysis by HPLC or LC/MS (122-124). The simplest is a liquid-liquid extraction. Extraction and chromatographic separation inevitably leads to some loss of analyte that can be corrected for by the inclusion of an internal standard.

Studies that have compared HPLC with other methods have shown that agreement of HPLC with LC/MS is close (125). Lensmeyer *et al* compared the correlation between HPLC and LC/MS as well as to a competitive binding assay on the Nichols Advantage system and an RIA from Diasorin. For HPLC, the correlation coefficient with LC/MS was 0.99. The Nichols Advantage overestimated 25(OH)D by 58% when 25(OH)D_3 was the predominant form and underestimated it by 27% when 25(OH)D_2 was the predominant form (126). The advantages of HPLC are its utility in resource-constrained settings, as the equipment is considerably cheaper than LC/MS, and it requires less expertise. The disadvantages of HPLC include the requirements for relatively large sample volumes, often a minimum of 500 ul and the long throughput times.

Immunoassays are commonly used to measure 25(OH)D. The advantages of immunoassays are that they are automated and can easily be incorporated into routine clinical chemistry practice. The immunoassay technique requires that 25(OH)D is first released from DBP, upon which the DBP is inactivated or precipitated. Immunoassays reportedly use a small volume (50ul or less) of sample; however, the dead volume required within the auto analyser is often considerably higher.

There are a number of issues pertinent to 25(OH)D assays that will be discussed in some detail, in below.

Assay Performance and Standardization

A publication by Binkley *et al* (2004) (127) was the first to highlight the variation in results obtained between the different methods used for the measurement of 25(OH)D. This publication emphasized the need for a reference standard material and the National Institute of Standards and Technology (NIST) working together with a number of other institutions then developed a standard, SRM 972, which encompasses four levels of standard. Level one is made from human serum, level two was diluted with horse serum to achieve lower levels of $25(OH)D_2$, level three was spiked with $25(OH)D_2$ and level four was spiked with 3-epi-$25(OH)D_3$. The hope was that this would improve inter-assay comparability, however, in a later study that compared automated immunoassays with LC/MS the bias for automated assays was shown to range from 0.5nmol/L to 11.4 nmol/L (128). Clinically this means that results can vary from 29 nmol/L, in the deficient range, to 40nmol/L, which is within the normal range. The classification of a patient as vitamin D sufficient or insufficient may therefore depend purely on the methodology used.

Benefits of the introduction of reference material and commercially available calibration material are evident by the considerably improved inter-laboratory coefficients of variability (CV) for LC/MS methods (129, 130). Data from DEQAS showed that about 10% of all methods for vitamin D testing used LC/MS. LC/MS methods had a positive bias of about 11% in comparison to the NIST LC MS/MS assay. Carter et al attributed this high positive bias to the fact that the NIST assay can resolve $25(OH)D_3$ from the 3-epi-2%$(OH)D_3$ which most LC MS/MS methods do not (131).

Matrix Effects

Matrix effects are a problem with immunoassays. The lipophilic nature of 25(OH)D makes it particularly vulnerable to the presence of lipids in the serum or plasma. These affect the ability of the binding agent to bind 25(OH)D equally in the sample and in the standard. Another problem is that 25(OH)D cannot be measured until it is removed from DBP. In a study of the effect of varying concentrations of DBP, researchers showed that five out of the six immunoassays evaluated showed an inverse relation between DBP concentrations and deviation from the gold standard

isotope dilution/online solid-phase extraction liquid chromatography/tandem mass spectrometry (ID-LC/MS/MS) methods (132). This has particular significance in the assessment of patients such as pregnant women and ICU patients. Pregnant women have high levels of DBP, which interfere with the efficacy of several of the automated assays. ICU patients on the other hand, have low levels of DBP, which may be one of the reasons for the low levels of 25(OH)D reported in ICU studies.

With LC/MS methods, the presence of co-eluting substances may lead to matrix effects such as ion suppression, which can in turn affect accuracy and precision. Furthermore, protein precipitation, which is used to displace 25(OH)D from its binding protein does not eliminate phospholipids or other matrix constituents. LC/MS methods vary with some employing only protein precipitation, while others use additional liquid-liquid extraction or solid-phase extraction (133). A significant advantage of using LC/MS is that the internal standard, needed for the correction of extraction losses and for the influence of ion suppression can be chosen so that it is chemically almost identical to the analyte of interest, by using stable isotope labelled standards.

Assay Cross Reactivity

More than 40 25(OH)D metabolites have been identified and any one of these may cross react in assays (134). In a comparison of LC/MS methods with immunoassays, several immunoassays showed variable measurement of $25(OH)_2D$ (135). Some assays e.g. the Abbot assay underestimates $25(OH)_2D_2$ while the Siemens assay overestimates $25(OH)_2D_3$ (136). This is problematic when vitamin D_2 is used for supplementation, as often happens in state hospitals in South Africa. Another problem is that some metabolites may circulate in concentrations that could interfere with 25(OH)D. Thus, $24,25(OH)_2D$ is found in serum in concentrations that are 10-15% of the circulating 25(OH)D levels. Furthermore, metabolite identification by immunochemical methods is challenging (137), but can be quantitated separately by LC/MS methods (138, 139). 25(OH)D is also metabolized through the C3-epimerization pathway (140). While the biological relevance of this remains to be determined, the C3 epimer is of interest, because it appears to be high in the serum of infants and may make a significant contribution to measured 25(OH)D levels in most LC/MS methods (141) but reportedly does not interfere with immunoassays.

The choice of a suitable method to measure 25(OH)D is challenging given the wide range of methodologies available. Even with the introduction of reference material and a reference method, standardization of immunoassays is not likely in the near future.

Given their cost, convenience and speed, automated immunoassays will likely remain the method of choice for African laboratories. Most automated assays report a functional sensitivity of 17.5nmol/L and as severe 25(OH)D deficiency is defined as levels <30nmol/L, a functional sensitivity at this level would be perfectly satisfactory.

1.7 DEFINITION OF VITAMIN D DEFICIENCY

The diagnosis of vitamin D deficiency based on the plasma concentration of 25(OH)D and this is used on a public health basis to consider the adequacy of vitamin D supply. Investigators have used different cut off levels to define vitamin D deficiency and adequacy (142-144). Thresholds to define deficiency and insufficiency have sometimes been based on levels at which 25(OH)D maximally suppresses PTH. Using this end point, investigators have reported cut off values ranging from 18nmol/L to 100nmol/L with a cluster around 80nmol/L (145-148). In 2010, the Institute of Medicine (IOM) issued a report on dietary reference intakes (DRIs) for calcium and vitamin D (131). The IOM stated that people with a serum 25(OH)D <30nmol/L are at risk of vitamin D deficiency as defined by skeletal disease, persons are potentially at risk at levels from 30nmol/L to 50nmol/L and practically all persons are vitamin D sufficient at levels >50nmol/L. The IOM stated that 50nmol/L was the estimate of the concentration that covered the needs of 97.5% of a normal healthy population in relation to bone health. The Endocrine Society released guidelines 6 months later, which defined vitamin D deficiency as a serum 25(OH)D value of <50nmol/L (149). They based this cut off on three main premises:

1) Elevation of PTH levels when 25(OH)D levels drop below 50nmol/L
2) Reduction in falls among older people on 800IU of vitamin D compared with those on 400 IU
3) Increased calcium absorption with 25(OH)D levels above 50nmol/L

The use of PTH in defining optimal vitamin D status has limitations, as increases in PTH occur with age (150), obesity (151, 152) and declining renal function (153).

The use of different cut offs has a huge effect on estimates of deficiency prevalence. Thus, data from the National Health and Nutrition Examination Survey (NHANES) showed that changing the definition of vitamin D deficiency from <27.5nmol/L to <50 nmol/L, increased the prevalence of vitamin D deficiency from 2% to 14% (154). Furthermore, as discussed previously the variability in the results from different methods for measuring blood levels of 25(OH)D causes considerable difficulty in the interpretation of results.

1.8 WORLDWIDE VITAMIN D STATUS

Reports from across the world suggest that vitamin D deficiency is re-emerging as a major global public health problem. In the following sections, I have summarized the available data; however, it is almost impossible to make direct comparisons across studies due to differences in the cut-off levels used to define vitamin D deficiency as well as the variation that arises from methodological differences.

North America (US and Canada)

In the United States, serum 25(OH)D levels have been assessed in participants in the NHANES over the periods 1988-1994 and 2002-2004(12). This is a national survey of the US population randomly selected from households in 81 counties across the United States. Comparisons of prevalence across regions are confounded by the fact that data collection and sampling occurred during the winter months in the lower latitudes and during the summer months in higher latitudes. Serum 25(OH)D was assayed in a central laboratory by radioimmunoassay after extraction with acetonitrile.

Vitamin D deficiency was defined as serum 25(OH)D of less than 25nmol/L and inadequacy as levels less than 30 to less than 62.5nmol/L. About one quarter of the population was at risk of inadequacy and 5% at risk of deficiency. The season-adjusted prevalence differed by sex and age and ranged from1- 8% in males to 1-12% in females.

In males as well as in females the prevalence of deficiency was lowest in those aged 1-8 years and increased until the age of 30 years in males and 18 years in females. Prevalence of those at risk of deficiency also varied by ethnicity and was greatest in non-Hispanic blacks (32%) followed by Hispanics (9%) and was lowest in non-Hispanic whites (3%). Initially it was reported that mean serum 25(OH)D decreased from 75nmol/L (95% CI, 72.5-75nmol/L) for the 1988-1994 collection to

60nmol/L (95% CI 57.5-62.5 nmol/L) for the 2001 to 2004 collection. However, it was later reported that the manufacturer had made some changes to the assay that was used for the measurement of serum 25(OH)D levels (155). These included the use of a new antibody with improved binding as well as the addition of detergents to a buffer to reduce non-specific binding. After a statistical adjustment, so that data from both collections could be compared (156) the age and season-adjusted prevalence of deficiency increased between 1988-1994 and 2001-2002 among males (3% to 8%) and females (7% to 11%). This clearly illustrates how assay differences confound the literature. Within NHANES it was subsequently recommended that the LC/MS method developed by NIST serve as a higher order reference measurement procedure and the need for a commutability study for the NIST SRM 972 was noted (156).

To analyse possible reasons for lower vitamin D status in the NHANES cohort in 2001-2006 relative to 1988-1994 Ganji *et al* looked at changes in BMI, milk consumption and physical activity in the group (157). They reported that mean BMI increased in all the subsets studied, milk consumption increased in non-Hispanic blacks only while physical activity increased in non-Hispanic blacks. The increased prevalence of vitamin D deficiency was attributed to increased adiposity.

There are less data from Canada compared to the US. The Canada Health Measures Survey collected physical measures of health and wellness as well as blood and urine samples from over 5000 people at 15 sites spread across five regions of Canada (158). The 25(OH)D was measured by the Diasorin chemiluminescent assay. Using a cut-off level of 27.5nmol/L, an estimated 4% of the population were vitamin D deficient. Mean 25(OH)D level was 67.7nmol/L and with higher levels in females. Overall, the pattern of 25(OH)D levels followed a U shaped distribution: highest among children and those above 60 years and lowest between the ages of 20-39 years. Higher 25(OH)D levels were associated with white racial background, increased milk consumption and blood collection from April to October. Some of these findings were reproduced in the Canadian Multicentre Osteoporosis Study, (159) which is an on-going longitudinal population based cohort study in nine Canadian cities. Data from almost 2000 men and women showed that 2.3% of the population had 25(OH)D of less than 27.5nmol/L. Women were over-represented in this study and the mean 25(OH)D levels were not significantly different to levels observed in males when stratified by age. The

strongest predictors of lower 25(OH)D concentrations were winter and spring seasons, non-white race, BMI ≥ 30kg/m^2 and lower vitamin D supplementation.

South America

Data on the vitamin D status in Latin American studies are scarce and there are no population studies from this region. An international epidemiological investigation conducted in postmenopausal women with osteoporosis, found a high prevalence of hypovitaminosis in Mexico (67.1%), Brazil (42.4%) and Chile (50.4%) using a cut off level of <75nmol/L (43). At a cut-off of 37.5nmol/L, the prevalence was still considerably high at 8.1% in Mexico, 6.0% in Brazil and 7.8% in Chile. 25(OH)D was measured at a central laboratory using the Nichols Advantage competitive binding chemiluminescence immunoassay.

Unger *et al* investigated the vitamin D status of 603 healthy male and female volunteers aged between 18 and 80 years in Sao Paulo Brazil (93). Bloods were collected after winter and a subset of 209 bloods were collected a second time, after summer. Marked seasonal variation in 25(OH)D levels was noted. Median winter 25(OH)D levels were 55nmol/L (41.5-72.3) and summer levels were 85nmol/L (65.5-109). The prevalence of vitamin D deficiency, as defined by 25(OH)D levels of less than 37.5nmol/L was 13.8% in winter as compared to 3.8% in summer. Other predictors of 25(OH)D levels were age and skin colour.

Differences in 25(OH)D levels were attributed to altitude and skin colour in a cross sectional study carried out amongst two groups of boys in Argentina (160). The prevalence of vitamin D deficiency (<25nmol/L) in Indian boys with dark skin, living in a mountainous region was 51.2% as compared to 0.9% in a group of mixed population boys living in Buenos Aires. Another possible contributor to lower levels in children from the mountainous area is the use of increased clothing to keep warm, although the investigators did not discuss this. A study from Sao Paulo, Brazil, in apparently healthy girls revealed mean 25(OH)D levels to be similar to the Argentinian study at 53.2 ± 17.0 nmol/L. There was no seasonal variation in 25(OH)D levels (161).

Europe

The vitamin D status within different European countries shows wide variation and, as in other regions of the world, varies with season, latitude and skin pigmentation.

Both the United Kingdom Low Income Diet and Nutrition Survey and the United Kingdom National Diet and Nutrition Survey showed that season, skin colour, dietary intake of vitamin D and intake of dietary supplements were significant predictors of 25(OH)D (162). Of particular interest was the observation that the low-income group had lower 25(OH)D concentrations than the general population. In addition to the season and use of supplements, 25(OH)D concentrations were lower in northern parts of the United Kingdom compared to the southern parts. 25(OH)D levels were highest in September. Women had lower levels than men in summer and autumn but not in winter.

A Norwegian study showed 25(OH)D levels to be higher (74.8 ± 23.7nmol/L) in those born in Norway compared with people of Pakistani origin living in Oslo (25.0 ± 13.6nmol/L) (163). While the reasons for these differences were not investigated, the high levels in indigenous Norwegians were attributed to consumption of oily fish and fortification of foods with vitamin D. The levels of 25(OH)D noted for Norwegians are in contrast to those from a population-based survey in Finland which showed the population mean (± SEM) 25(OH)D level in men to be 45.1 ± 0.53 nmol/L and this was not significantly different to levels in women(164). Both Norway and Finland are at approximately the same latitude, 60^{o}N. It is possible that some of these differences are due to differing assay methodology as well as season of draw. All bloods were drawn in the winter months of January and February for the Finnish study.

Table 1.4: European Studies of 25(OH)D Levels

Country	Reference	Population and (N)	25(OH)D assay	25(OH)D nmol/L
Norway	(163)	Norwegians (869) Pakistanis (177)	Diasorin RIA	74.8 ± 23.7 25.0 ± 13.6
Finland Finland	(164) (165)	Finnish men and women aged 62-79 (1604) Bangladeshi(34), Somali(48) and Finnish(61) premenopausal women	RIA ELISA	45.1 ± 0.53* Bangladeshi 42.9 ±16.1 Somali 36.8 ± 11.8 Finnish 54.1 ± 19.1
Italy	(166)	Italian premenopausal women(608)	ELISA	Northern sites 69.0 ± 27.8 Central sites 72.3 ± 28.3 Southern sites 72.0 ± 28.0
France	(167) (168)	Premenopausal women from several countries including France(637) Osteoporotic and osteopaenic women (1292) age 52-94 years	Diasorin RIA	87 ± 2.20* 51.5 ± 26.1
Spain	(169) (170)	Male and female adults ages 18-84 years(177) Male and female adolescents (100)	Chemiluminescent assay ELISA	Males 61.3 ± 22.9 Females 59.5 ± 21.9 63.6 ±18.9
Germany	(171)	Men and women(4030)	Diasorin chemiluminescent	Men 45.2 (30.5-68.7) Women 44.7 (30.7-72.7)
The Netherlands	(172)	Dutch men and women Turkish men and women Moroccan (613)	Diasorin RIA	Dutch 67 (50-83) Turkish 27 (18-29.5) Moroccan 30 (20-45)

RIA=radioimmunoassay; ELISA= enzyme linked immunosorbent assay; *values are mean ± SEM whilst all other values are mean ± SD or median (interquartile range)

What is obvious is that most European countries lack large population studies that have investigated the prevalence of vitamin D deficiency. It is interesting to note that several studies demonstrate lower levels of 25(OH)D in immigrant communities (163, 165, 173, 174). The reason for this observation has not been investigated, but may include darker skin colour, and cultural practices such as dress style.

Asia

Apart from Korea, there are no Asian population studies. There are several studies from China, but I have selected only studies with very large sample sizes numbers. Small studies have been included if they were performed in India. These are summarised in table 1.5.

Table 1.5: Asian Studies of 25(OH)D Levels

Country and Reference	Population and (N)	25(OH)D nmol/L	Determinants of lower 25(OH)D
Korea (KNHANES) (175)	Age >10 years. Males and females, population survey(6925)	Males 53.0 ±18.8 Females 45.5 ±17.8	Winter, spring, urban residence and indoor work.
India (176)	Male and female young adults ages 18-35 years(329)	Summer 132 ± 84.3 Winter 78.5 ± 53.0	Season, duration of sun exposure in winter , calcium intake
Rural India (177)	Males and females (23)	36.4 ± 22.5	Higher levels in rural populations compared with literature for urban populations
India (178)	Males and females rural vs. Urban(1143)	Males rural 59.3 ± 2.0* Males urban 46.3 ± 2.0* Females rural 47.5 ± 2.2* Females urban 38.8 ± 0.8*	Higher levels in rural populations.
India (179)	Pregnant women and Adolescent girls (288)	Pregnant women 37.8 ± 19.8 Adolescent girls 33.3 ± 16.0	Female gender associated with lower 25(OH)D
India (96)	Pregnant women and then women and their infants(541)	Looked at three trimesters as well as seasonal effect 1st trimester summer 23.4 ± 11.3 2nd trimester summer 25.7 ± 15.1 3rd trimester summer 27.7 ±9.7	There was no significant change in 25(OH)D levels across trimesters but 25(OH)D levels were lower in winter across all trimesters. Strong positive correlation of maternal 25(OH)D with infant 25(OH)D
India (180)	Healthy adults aged 50 years and above(1346)	Divided group into those 50 to 65 and then above 65	Aged 50-65 35.2 (0.77,249) in Aged > 65 34.8 (4.04,230) VDD defined as 25(OH)D <50nmol/L was present in 91.2% of all subjects with no significant difference in both groups.
Pakistan (181)	Males and females(300) Ages 38-55 years	47.9 (31.5, 61.5)	Use of vitamin D supplements
Japan (182)	Women age >74 years	59.9 ±17.9	Fish intake
China (183)	Men and women (25988)ages 20-96years	52.2 (42.3, 62.5) 30% males and 46% females had 25(OH)D <50nmol/L	All samples taken during winter. Men had significantly higher 25(OH)D levels than women
China (184)	Pregnant women(1695) Mean age 28.1 years	43.9 ± 28.6 69% of subjects had 25(OH)D <50nmol/L	25(OH)D correlated negatively with age, body weight and BMI

*values are mean ± SEM whilst all other values are mead ± SD or median (interquartile range)
VDD= Vitamin D deficiency

The results of Asian studies highlight gender differences in 25(OH)D levels. The implications of low maternal 25(OH)D levels for infant and child health are important to follow up, perhaps particularly so in female infants as girls have

significantly lower levels than boys. It is important to note that 25(OH)D levels are higher in rural Indian populations than in urban populations and are lower in those who work indoors. This is to be expected, particularly where dietary intake of vitamin D is low and is especially relevant because of increased rural to urban migration.

Australia

Although Australia has a sunny climate, low mean 25(OH)D levels have been reported from certain high-risk groups such as women living in residential care (185). Marked seasonal variation was noted from a longitudinal study in an elderly population from Tasmania with mean levels in winter of <50nmol/L. Also shown is that supplementation with 25(OH)D reduced the winter drop in 25(OH)D concentrations (186). A large population based study noted that the prevalence of vitamin D deficiency defined as a level of <50nmol/L was 31% with a higher prevalence in women (39%) compared to men (22%) (187). Predictors of low levels were increased age, non-European origin, living in southern Australia, female gender, obesity and physical inactivity.

Africa and the Middle East

There is a paucity of population-based studies from Africa and the Middle East. Lack of sunshine exposure and veiled clothing style are important factors that influence hypovitaminosis D (188). A study from Saudi Arabia reported that 79.1% of females have 25(OH)D levels of <25nmol/L which is in the deficient range (189). A high prevalence of vitamin D deficiency has also been noted in Saudi men, in which lower levels of 25(OH)D were noted to be associated with increased age and obesity (190). However, adequate 25(OH)D levels have been reported from a number of sub-Saharan countries. Pregnant HIV infected women from Tanzania were noted to have mean levels of 89.2nmol/L, while a study from Uganda showed a high level of vitamin D sufficiency as defined by levels >75nmol/L in HIV infected individuals, with and without tuberculosis (191).

There are no population-based studies from South Africa. A study of 10-year old children from Johannesburg showed that mean 25(OH)D levels were high and that deficiency was rare (192). This study showed that white children had significantly higher levels than their black peers had (120-±36 nmol/L vs. 93.3 ± 34nmol/L). Winter season, female gender and increased fat mass were all associated with lower 25(OH)D levels. Alcohol use was shown to be a determinant of lower

25(OH)D levels in adolescents from Tygerberg (193). Kruger *et al* (2011) investigated risk factors for low bone mineral density (BMD) in rural as well as urban African women and noted that women older than 50 years who lived in an urban area had lower 25(OH)D levels compared to their rural peers (194). The prevalence of vitamin D deficiency defined as a level less than 37.5nmol/L was noted to be 41% in a retrospective analysis of 25(OH)D laboratory results at Tygerberg hospital (195). In another Cape Town study, marked seasonal variation of 25(OH)D levels was noted, with highest levels observed from January through to March (56nmol/L vs. 30.7nmol/L) (92).

In summary, while there is no uniform definition of vitamin D deficiency, the lowest levels are noted in immigrant populations in Europe as well as in African-Americans. Cultural factors such as mode of dress are associated with high prevalence of deficiency even in very sunny countries like Saudi Arabia. There are very few studies that have looked at levels in South African adults, in particular South African men and none that have reported on 25(OH)D levels in the Indian population in South Africa, a group noted to be at high risk for deficiency from studies across the world. Studies from India and South Africa have highlighted differences in 25(OH)D levels of rural populations compared to urban populations and this raises concerns for a nation whose population is in transition.

1.9 CONSEQUENCES OF VITAMIN D DEFICIENCY

The classical effect of vitamin D deficiency is recognized as failure of normal mineralization of the bone. This presents as rickets in childhood and as osteomalacia in adults. In both these conditions, there is poor mineralization of the bone matrix so that bones are soft. The hallmark of severe vitamin D deficiency is a low circulating level of 25(OH)D.

Vitamin D levels of between 50-125nmol/L are found in the majority of studies conducted among healthy children in communities where vitamin D deficient rickets is uncommon (196, 197). The classical biochemical changes in vitamin D deficient individuals are hypocalcaemia, hypophosphataemia, elevated alkaline phosphatase and PTH. Florid rickets manifests with leg deformities, enlargement of the growth plates of the wrists, ankles and costochondral junctions and rib cage deformities (198). X-rays confirmation of the impaired mineralization of the growth plate as evident by widening of the growth plate and fraying of the margins of the metaphyses is required to make the diagnosis.

The clinical manifestations of osteomalacia differ from those of rickets, and often include bone pain mainly between the joints as well as proximal muscle weakness and gait instability. As the growth plates in adults have already closed, radiological features are different. X-rays may reveal pseudo fractures of the pelvis, femurs, metatarsals or lateral margins of the scapula (199). In addition to the effects of vitamin D on bone, a large number of studies suggest that it has other skeletal and non-skeletal benefits.

1.9.1 The Effects of 25(OH)D on Bone Mineral Density

Bone physiology is dependent on the activities of osteoblasts, osteocytes and osteoclasts. Both osteoblasts (which are bone-forming cells) and osteocytes are derived from the mesenchymal cell lineage. Conversely, osteoclasts (which are bone resorbing cells) are derived from the haemopoetic lineage. Each of these three cell types express the VDR and are capable of metabolizing 25(OH)D to $1,25(OH)_2D$ to exert biological activity(200). Current data indicate that vitamin D acts to maintain plasma calcium and phosphate levels and prevent the development of rickets or osteomalacia. Calcitriol acts on osteoblasts to maintain osteoclastogenesis and bone resorption which are associated with failure of bone mineralization. On the other hand there is on-going debate about the association between 25(OH)D and BMD. Common allelic variations in the gene encoding the vitamin D receptor have been associated with increased bone density in healthy individuals (201). *In vitro* studies of VDR over expression on mature mouse osteoblasts were associated with an inhibition of bone resorption (202). Conversely, animal models have shown that 25(OH)D levels are inversely related to trabecular bone mineral volume (203). $1,25(OH)_2D$ appears to affect both osteoblast and osteoclast function. *In vitro* experiments with rodent or human primary bone cells show that the inclusion of $1,25(OH)_2D$ in the culture media inhibits osteoblast proliferation and enhances osteoblast maturation and mineral deposition (200, 204). Genes coding for the vitamin D metabolising enzymes CYP27B1 and CYP24A1 are expressed in several cell types including osteoblasts and osteoclasts (205). Expression of these genes is positively related to each other and is unrelated to serum $1,25(OH)_2D$ concentration, suggesting that locally produced $1,25(OH)_2D$ may have an autocrine or paracrine effect on bone. In support of this concept, expression of bone cell CYP24A1 is not related to serum $1,25(OH)_2D$ concentrations. 25(OH)D metabolism appears to promote osteoclast

differentiation and reduce osteoclast activity (206). Thus, 25(OH)D may act on bone by altering the activity of both osteoblasts and osteoclasts.

Cross sectional studies of the relationship between BMD and 25(OH)D have yielded conflicting results with some studies showing BMD to be independent of 25(OH)D (207-210), while others have shown a positive association between 25(OH)D and BMD (211, 212). Two systematic reviews concluded that there was insufficient evidence of an effect of vitamin D supplementation on BMD (213, 214). There are several possible reasons for these conflicting results: small sample sizes, different population groups and different covariates used in multiple regression analysis. Furthermore, BMD is dependent on several other factors including hereditary factors, body weight, medications, age and lifestyle (215-219). Few South African studies have reported on 25(OH)D and BMD. Daniels et al (1997) showed that there are ethnic differences in BMD between Africans and Caucasian females with Africans having greater femoral neck BMD compared to Caucasians. 25(OH)D levels were lower in Africans and the greater BMD was explained by greater weight bearing in Africans (220).

A consistent finding from observational studies is that vitamin D insufficiency and not necessarily deficiency, increases fracture risk (221). The majority of hip fracture patients demonstrate osteoporotic bone histology, meaning that they have a reduced ratio of mineral to osteoid (222). Results of some randomised controlled trials (RCT) of vitamin D supplementation, particularly when combined with calcium supplementation, demonstrate reduced fracture risk (223, 224).These findings implicate reduced vitamin D as a cause of osteoporosis.

In a systematic review and meta-analysis of fracture risk in relation to vitamin D supplementation and 25(OH)D levels, Lai et al noted 33% lower 25(OH)D levels in cases compared to controls, while the results of seven RCTs showed no difference in hip fracture risk in those randomized to supplementation versus placebo (221). In addition to the reasons for a lack of association listed above, it is possible that the doses of vitamin D used in RCTs were insufficient or that the form of supplementation was unsuitable. Results of a meta-analysis by Bischoff-Ferrari *et al* showed that vitamin supplementation at doses of between 700 and 800IU per day reduced the relative risk of hip fractures by 26%, versus calcium or placebo, while doses of 400IU/day did not show any significant benefit (225). Different inclusion criteria were used for these meta-analyses. Lai et al (221) excluded trials

that included combined therapy, while Bischoff-Ferrari included vitamin D supplementation and combination therapy (225). Another reason for a lack of association may be the form of vitamin D used therapeutically. Cholecalciferol or ergocalciferol will not be activated in 25(OH)D replete patients or in those with impaired renal function e.g. elderly patients. In contrast, calcitriol or the pro-drug alfacalcidiol will act independent of the patient's vitamin D status or kidney function. An early meta-analysis of trials that included over a 1000 patients showed a 22% reduction in falls in subjects receiving vitamin D supplementation (226). In depth analysis of this meta-analysis revealed these results were driven by two trials that had used calcitriol or alfacalcidiol and these studies supplied up more than half the total subject population.

Data from a nested case–control study within the Women's Health Initiative (WHI) showed that ethnic differences also contribute to disparities in study results. In white women 25(OH)D levels greater than 50nmol/L compared to levels lower than 50nmol/L were associated with lower risk of fractures, while in African–American women levels of 25(OH)D of greater than 50nmol/L compared with levels less than 50nmol/L were associated with greater fracture risk (227). They suggest that this is evidence for different 25(OH)D threshold requirements for different ethnicities. Other causes for increased fracture risk could be increased secretion of PTH in vitamin D deficiency which has been well described and can contribute to bone fragility and fractures in the elderly through increased bone turnover and decreased bone density (148, 228). The threshold 25(OH)D levels required to suppress a rise in PTH levels appears to vary by age, gender, adiposity and race (151, 152, 229).

Finally, fracture risk may be due to muscle weakness, which affects gait. Muscle weakness and hypotonia are features of rickets and osteomalacia. Adults may complain of pain, which may be prominent about the hips and may produce a waddling gait. This muscle weakness is multifactorial and includes reduced calcium uptake into the sarcoplasmic reticulum (230), impaired muscle contraction and muscle atrophy (231). In addition, muscle cells have VDRs and the expression of these decreases with advancing age (232). 25(OH)D levels that are often lower in the elderly may modulate fracture risk by decreasing falls via effects on muscle.

1.9.2 Vitamin D and Cardiovascular Risk Factors

Epidemiological evidence that has shown that low levels of 25(OH)D are associated with increased risk of cardiovascular disease including hypertension and myocardial infarction (233, 234). Laboratory evidence confirms this, as the VDR appears to be widely distributed in several tissues including the heart and blood vessels. VDR deficient mice develop hypertension, cardiac hypertrophy and increased thrombogenicity (231). 25(OH)D may influence glucose control as pancreatic β cells have both CYP27B1 and the VDR and 25(OH)D is involved in both insulin synthesis and release (235). $1,25(OH)_2D$ is thought to affect glucose tolerance as it has been shown to be involved in normal insulin secretion and reduced concentrations have been associated with β cell dysfunction (41), insulin receptor down regulation and insulin resistance (236). In addition, $1,25(OH)_2D$ may protect islet cells from cytokine mediated apoptosis (237) and has been shown to blunt the impact of advanced glycation end products on endothelial cells (238) Furthermore, low levels of 25(OH)D have also been associated with unfavourable lipid levels which may lead to increased cardiovascular risk (239).

In the following section I will review some of the studies on associations between 25(OH)D and dysglycaemia, serum lipids and hypertension.

1.9.3 Vitamin D and Type 2 Diabetes

There is ample laboratory evidence for a role of vitamin D in insulin secretion such as the presence of VDR in pancreatic B cells (240, 241). Vitamin D deficient rats have glucose intolerance and altered insulin sensitivity(242). Mechanism may involve a rise in intracellular calcium concentrations (243) or insulin exocytosis (244). While there is mechanistic evidence for a role of vitamin D on pancreatic B cell function the results of clinical studies are not clear-cut and are reviewed below.

Results of Cross Sectional Studies

The initial observation for the relationship between vitamin D and type 2 diabetes (T2DM) in humans came from studies showing that both healthy and diabetic subjects experienced seasonal variation in glycaemic control (245, 246). Since then some studies have shown a similar association between 25(OH) D and T2DM or glucose intolerance, while others have failed to show the same relationship. Results of some of the large cross sectional studies (N numbers > 1000), that have examined associations between 25(OH)D and blood glucose levels, insulin

secretory capacity or insulin secretion are summarised in Table 1.6. Smaller studies have been included if they included Indian subjects.

Table 1.6: Studies on the Association between 25(OH)D and Type 2 Diabetes Mellitus or Metabolic Syndrome

Study Type (Reference)	(N) Ethnicity	Participant Type Age Males (%)	Outcome
NHANES III-cross sectional (247)	(6228) Mixed No Asian Americans	Healthy, excluded diabetics ≥ 20 years (47)	OR for diabetes varied inversely across quintiles of 25(OH)D in NHW (OR 0.25 (CI 0.011-0.60)) and MA (OR 0.17 (CI 0.08-0.37)), for those in the highest quintiles compared with those in the lowest (≥81.0nmol/L vs≤43.9 nmol/L). This was not seen in NHB. HOMA was inversely associated with 25(OH)D in NHW (p= 0.058) and MA (p=0.0024). This was not seen in NHB.
Cross sectional (248)	(3262) Chinese	Used all participants 50-70 years (44)	Aim was to evaluate association between 25(OH)D and Met S. Showed an inverse association between 25(OH)D and fasting insulin (β=-0.06, p=0.01), and HOMA index only in overweight subjects (β=-0.06, p=0.004).
NHANES cross sectional (249)	(9773) mixed	All participants ≥ 18 years	Aim was to look at the association of 25(OH)D with HbA1c. They showed that 25(OH) D was inversely associated with HbA1c only in those between 35 and 74 years (p=0.004) and those who did not report a history of diabetes. PTH was also inversely associated with HbA1c (p=0.0002) in those aged 35-74 years.
Cross sectional (250)	(5787) Korean	Used all subjects ≥ 20 years (42)	25(OH)D inversely associated with fasting glucose. Compared to individuals with a sufficient serum 25(OH)D concentration ≥75 nmol/L, the OR (95% CI) for diabetes mellitus were 1.73 (1.09–2.74), 1.30 (0.91–1.84), and 1.40 (0.99–1.98) for serum 25(OH)D concentrations <25, 25 to <50, and 50 to <75 nmol/L, respectively, after multiple adjustments (*P*-trend < 0.0001).
Case control (251)	(210) South Asian	170 subjects with diabetes, 40 without. >40 years (50)	Diabetics recruited from diabetic clinic. There was no significant difference in 25(OH)D levels between those with T2DM and those without, however, vitamin D deficiency was more common in those with T2DM (83% vs. 70%, p=0.07)
Cross sectional (252)	(2465) Caucasian	58-71 years (53) Self-reported diabetes Fasting blood sugar or OGTT not done	Looked at OR for self-reported diabetes in subjects with 25(OH)D ≥80 nmol/L vs. ≤ 37 nmol/L (OR=0.5(CI 0.1-0.7))
Cross sectional (253)	(441) Asian Indian	37-53 years (54) Randomly selected Indian living in India	Lack of association between 25(OH)D and HOMA-IR (β=-0.12; p=0.17), fasting glucose (β=0.26; p=0.79) and insulin (β=0.14; p=0.89)

Table 1.6 continued

Cross sectional (254)	(1017) Caucasian	30-54 years (32)	PTH and not 25(OH)D was associated with the Met S. Subjects with PTH in the 2nd to 4th quartiles had the highest OR for Met S 2.33 (95% CI 1.40-3.87). PTH correlated with blood pressure but not with fasting blood glucose or HOMA-IR.
Cohort (255)	(1070) Majority Caucasian	74.5± 9.5years for men 74.6 ± 10.7 years for women (38) Cohort: these were results from a follow up study	No evidence for association of 25(OH)D with Met S, Significant trend (p=0.03) for adjusted odds for Met S with increasing PTH (OR 2.02 (CI 0.96-4.24)). This was only seen in men and was largely due to effects on blood glucose and low HDL.
NHANES Cross sectional data (256)	(3206) Mixed	≥ 20 years (49) Excluded physician diagnosed diabetes	After adjusting for BMI, abdominal obesity hyperinsulinaemia, HOMA-IR and 2-hour OGTT, risk for hyperglycaemia decreased across quintiles of 25(OH)D. Hyperinsulinaemia was positively associated with PTH.
Korean National Health and Nutrition Survey (257)	(5559) Korean	≥ 19 years (42) Used all subjects	After adjusting for confounders there was no association between 25(OH)D and any of the components of the Met S. The association of PTH with Met S or its components was not reported.

Key: HbA1c haemoglobin A1c; HOMA-IR HOMA Insulin Resistance; Met S metabolic syndrome; OGTT oral glucose tolerance test; PTH parathyroid hormone; OR Odds Ratio; NHW Non-Hispanic Whites; NHB Non-Hispanic Blacks; MA Mexican Americans.

Almost all studies used 25(OH)D as a biomarker for vitamin D stores and the majority of studies listed in Table 1.6 show an inverse relationship between 25(OH)D and glycaemia. However, this relationship is not consistent. Possible reasons for differences may include failure of some investigators to adjust for BMI. Other reasons may reflect ethnic differences or differences in the mean 25(OH)D levels of subjects across studies. A number of studies have shown that after adjustment for confounders such as BMI, the relationship was no longer significant (258, 259). Furthermore, several of the studies failed to adjust for parathyroid hormone (PTH), which rises with obesity and is often elevated in vitamin D deficient states. Thus, the sequelae of vitamin D deficiency may be mediated by increased concentrations of PTH. PTH has been shown to be independently associated with hyperglycaemia, HOMA-IR and other components of the metabolic syndrome (254, 260) and parathyroidectomy has been shown to improve glucose homeostasis (261).

Results of Prospective Studies

The Nurses' Health Study followed 83779 women who had no history of cancer, diabetes or cardiovascular disease at baseline for the development of T2DM (262). They evaluated vitamin D and calcium dietary as well as supplement intake but did not measure 25(OH)D concentrations. Over 20 years, 4843 incident cases of T2DM were reported. They reported a clear inverse dose-dependent relationship between calcium intake and incident diabetes. The relative risk of diabetes was 0.87 (CI 0.75-1.00) when the highest level of vitamin D supplementation was compared with the lowest group (800IU vs. 400 IU). They also showed that women who took a combination of calcium plus vitamin D at a higher concentration of > 1200mg and >800IU respectively had a lower incidence rate of diabetes vs. <600 mg and <400IU.

A nested case control study of the same cohort (608 diabetics and 559 control subjects) looked at the association of baseline 25(OH)D and risk of incident diabetes (263). After adjusting for diabetes risk factors the OR for incident T2DM in the top versus the lowest quartile of 25(OH)D was 0.52 (CI 0.33- 0.83).

Another study that supports the inverse association of 25(OH)D with diabetes is the Women's Health Study (264). This included 10 066 women and assessed the impact of dietary and supplemental vitamin D and calcium on diabetes and cardiovascular disease. Serum 25(OH)D was not measured. There was a significant decrease in the cumulative incidence rate of diabetes from 4.6% in those with the lowest total intake of vitamin D vs. 3.4% in those with the highest intake of vitamin D (p for trend=0.02). They also showed a decrease in the incidence for diabetes from 5.5% for those with the highest intake vs. 2.7% for those with the lowest intake (p for trend <0.0001). The prevalence of other components of the Met S, hypertension, central obesity, low HDL cholesterol decreased significantly in those with the highest vitamin D intake, relative to those with the lowest vitamin D intake. After adjusting for age, smoking, total calories, alcohol use, multivitamin use and parental history of myocardial infarction, total vitamin D intake was no longer significant for the Met S (p=0.13).

The Australian Diabetes, Obesity and Lifestyle (AusDiab) study was designed to determine the prevalence (1999-2000) and incidence (2004-2005) of type 2 diabetes in Australia (265). Within an initial cohort of 11247 individuals who had an OGTT at baseline, 6537 returned for an OGTT at follow up. During the five-year

follow up, 199 incident cases of diabetes were diagnosed. Those who developed diabetes had lower mean serum 25(OH)D than those who did not develop diabetes (58 ± 23 vs. 65 ± 25nmol/L, p <0.001) as well as lower calcium intake. This was after adjusting for a number of covariates including age, ethnicity, physical activity and season. They also noted that higher serum 25(OH)D was associated with increased insulin sensitivity.

A Swedish study by Deleskog et al investigated whether serum 25(OH)D predicted the development of impaired fasting glucose, impaired glucose tolerance or T2DM in 2288 men and women aged 35-56 years and without diabetes at baseline (266). Follow up was for 8 to 10 years. Men but not women in the highest quartile of 25(OH)D had a decreased OR (0.52 (CI 0.30, 0.90)) for developing T2DM after adjusting for confounders including BMI and physical activity. This effect was only for individuals with either impaired fasting glucose or impaired glucose tolerance at baseline and not for those with a normal OGTT result. They did not report on associations between 25(OH)D and insulin resistance or insulin secretion. Mean 25(OH)D levels in all men and women were greater than 50nmol/L.

The importance of adjusting for BMI is highlighted in the results of the Tromso study (267). Over 6000 subjects, without diabetes, aged 25 and older were recruited, focussing initially on cardiovascular disease. Subjects were stratified as smokers or non-smokers. Subsequently diabetes was diagnosed if self-reported or based on HbA1c >6.5% in 247 subjects. Baseline 25(OH)D was inversely associated with T2DM, but after adjusting for BMI this association was no longer significant. The mean 25(OH)D level was adequate at 52.8 ± 16.8 nmol/L.

The Inter99 study, based in Copenhagen, Denmark, reported on the effects of lifestyle intervention for CVD. A random sample of 6405 men and women, aged 30-65 was used to assess the association between baseline 25(OH)D and the development of T2DM after five years follow up (268). Diabetes diagnosis was based on results of OGTT, or HbA1c of >6.5%, self-report or use of diabetes medication. Incident diabetes was diagnosed as diabetes at follow up among those who did not have diabetes at baseline. Median 25(OH)D level was 48nmol/L (IQR 12.0-118). Six hundred and seventy seven subjects had diabetes at baseline and a further 141 developed diabetes during follow up. Low 25(OH)D was associated with a higher prevalence of diabetes at baseline after adjusting for confounders including BMI and physical activity. Low 25(OH)D level was

significantly associated with incident diabetes in crude analysis but this became insignificant after adjustment for confounders. They also reported that low 25(OH)D was significantly associated with increased fasting and 2-hour blood glucose levels as well as insulin resistance.

Kirii et al examined the relation of calcium and vitamin D intake to T2DM risk in a large Japanese cohort (269) and reported that neither calcium nor vitamin D intake was independently associated with T2DM, but may have a joint protective action. A weakness of this large study was that they did not measure serum 25(OH)D or serum calcium and diabetes was self-reported.

A recent publication reported on the association between 25(OH)D at baseline and incident T2DM over a 29 year follow up period (270). They reported adjusted HR for type 2 diabetes of 1.35 (CI 1.09-1.66) for the lowest vs. the highest quartile of 25(OH)D. A major flaw of this study was the method used to diagnose diabetes. Diabetes was excluded at baseline if a non-fasting blood glucose was <11mmol/L and was diagnosed at end point either if the subject was on medication, self-reported or non-fasting blood glucose was >11mmol/L.

The only prospective study to date that had the development of diabetes as the primary end point was from Korea (271). An increased risk for type 2 diabetes was noted in those subjects with 25(OH)D levels of <25nmol/L after adjusting for a number of confounders including BMI, waist circumference and insulin resistance. One thousand and eighty non-diabetic Koreans with one or more risk factors for T2DM were recruited. The mean 25(OH)D level was 49 ± 23 nmol/L. Diabetes was diagnosed by HbA1c > 6.5%. They reported an increased risk of T2DM in subjects with vitamin D deficiency independent of BMI, HOMA-IR and pancreatic function.

In a meta-analysis of twenty one prospective studies that looked at the association between 25(OH)D levels and risk of incident T2DM, Song et al noted that there was an inverse association between 25(OH)D levels and risk of T2DM that was independent of 25(OH)D assay, sample size, gender or population type (272). They reported that this association did not differ by sex, sample size, method used to diagnose diabetes or 25 (OH) D assays. Nineteen out of 21 studies included predominantly white subjects.

A recent case control study from Tehran noted that baseline 25(OH)D levels contributed to T2DM rate in a non-linear fashion with a sharp increase in risk for 25(OH)D values below 25nmol/L (273).

Few studies examined the association of vitamin D with β cell function and glycaemia prospectively. Kayaniyil et al prospectively examined the associations of baseline 25(OH)D with insulin resistance, β cell function and glucose homeostasis in subjects at risk for T2DM (274). This was a mixed ethnic population recruited from London and Toronto with a mean age of 50.2 ± 9.7 years. They used a number of measures to determine insulin resistance, insulin sensitivity, whole body insulin sensitivity and β cell insulin function. Baseline 25(OH)D was 58.0 ± 23.2 nmol/L and did not show any association with insulin resistance or insulin sensitivity but reported a significant association with measures of β cell function and area-under-the-curve for glucose. A flaw in this study was that follow up 25(OH)D was not measured.

The results of most prospective studies therefore suggest that baseline vitamin D status may have an impact on future risk of T2DM. There is considerable heterogeneity in study design, population groups, mean 25(OH)D levels and adjustment for confounders and it is therefore important to consider the results of randomized control trials.

Results of Randomized Controlled Trials

There have been very few randomized controlled trials, and those that do exist have small numbers of subjects. George et al conducted a systematic review and meta-analysis of the effect of vitamin D supplementation on insulin resistance and glycaemic control (275). They included 15 trials in the systematic review. The randomized controlled trials used vitamin D vs. placebo, vitamin D and calcium supplementation vs. calcium supplementation alone and vitamin D and calcium supplementation vs. placebo. Outcomes were changes in fasting blood glucose, HbA1c, C-peptide, insulin resistance, development of microvascular complications or of macrovascular complications. Results of combining all studies showed that patients with impaired glucose tolerance or diabetes demonstrated a small reduction in fasting blood glucose (FBG) on treatment with vitamin D. The studies were small (276, 277), lasted short periods of time and used varying doses of vitamin D. Treatment had no effect on long term glycaemic control as determined

by HbA1c in those with diabetes and no effect on any outcomes in subjects with normal glucose tolerance (277-279). These studies were probably inappropriate to determine long-term glycaemic control as the duration ranged from 8 weeks to 6 months.

In summary, while data from cross sectional studies and observational studies are sometimes conflicting, in general, they suggest an association between low levels of 25(OH) D and T2DM, but this has not been confirmed by the results of randomized controlled trials. The few randomized controlled trials that were conducted have included very few subjects, especially those with abnormal glucose tolerance, and may therefore not be powered sufficiently to determine the effect of 25(OH) D on glucose control.

1.9.4 **25(OH)D and Serum Lipids**

Low serum levels of 25(OH)D have been associated with unfavourable lipid profiles that could possibly explain the relationship of 25(OH)D with cardiovascular disease.

This may be mediated via PTH or 25(OH)D. PTH has been reported to modify adipocyte function by inhibiting adipocyte lipoprotein lipase activity (280). Alternatively, 25(OH)D may alter lipid levels via effects on calcium. Zitterman et al suggested that 25(OH)D increases serum triglyceride (TG) levels by increasing serum calcium which then decreases hepatic TG formation or secretion (281). Moreover, high calcium intakes have been shown to increase faecal fat excretion, reduce fatty acid synthase mRNA expression and serum TG (282). Furthermore, vitamin D may affect insulin secretion and insulin sensitivity and thereby influence lipid metabolism.

It is unlikely that any effect seen is due to a single mechanism and as the VDR is widely distributed there may be direct effects via interactions between $1,25(OH)_2D$ and its receptor. However, the results of animal studies are conflicting, as one would expect VDR knock-out mice to have increased serum TG whilst they do not. Indeed, they have less body fat, less TG and less total cholesterol (TC) than wild type mice even when fed a high fat diet(283, 284). The reasons for this are not clear. One hypothesis is that $1,25(OH)_2D$ has a dose dependent effect, with low doses being stimulatory and high doses inhibitory (285, 286).

Results of Cross Sectional Studies of 25(OH)D and Serum Lipids

Some of the larger cross sectional studies, with 1000 or more subjects are summarised in Table 1.7. Of the studies listed in this table, five report a positive relationship between 25(OH)D and HDL-C (239, 248, 287-289). In contrast, the studies by Kim et al in Korean adults (257) Skaaby et al in Danish adults (290) Gagnon et al in Australian adults (291) and Hypponen et al in British adults showed no such association (292). It is possible that gender and ethnic differences account for these inconsistencies. The relationship between 25(OH)D and TG was consistently negative in the studies listed in Table 1.7. While the results of cross sectional studies do not prove causality, the consistently negative association between 25(OH)D and serum TG warrants further investigation.

Table 1.7: Cross Sectional Studies of 25(OH)D and Serum Lipids

Subjects (reference)	Age in Years mean ±SD	N (% males)	Lipids (direction of association with 25(OH)D)	Adjustment Other information
US adults White (234)	59±9	1739 (45)	+TC/HDL	No adjustments. Compared subjects 25(OH)D <37.5 to >37.5 nmol/L; p for TC/HDL <0.001.
Norwegian (239)	55.9±12.9	10105(45.5) (smokers and non-smokers)	+TC +HDL +LDL -TG, -LDL /HDL	Age, gender, BMI. Linear trends across quartiles of 25(OH)D. TG in the lowest quartile 1.62±0.80 vs. 1.32±0.81mmol/L in the highest quartile HDL-C 1.50 ± 0.40 vs. 1.59 ± 0.36mmol/L
Chinese adults (248)	50-70	3262 (44.2)	Males -TG +HDL NS Women	Age, gender, date, smoking, alcohol use, diabetes, BMI. MVR p<0.0001 for both TG and HDL-C in men.
Korean adults (257)	≥ 19	1330 (28)	-TG NS HDL	Age, gender, BMI, season, PTH, calcium intake and serum calcium. OR for TG across 25(OH)D quintiles 0.34 (0.20,0.56); p for trend <0.001
US adults NHANES Three cycles 2001-2002 2003-2004 2005-2006 (287)	>20	3958 (48.2)	+HDL NS TG	Results for 2554 subjects. Adjusted for age, gender, poverty-income ratio, waist circumference, smoking, PTH and calcium. Quintiles of 25(OH)D. Reported mean difference in z-score per 1 SD difference in 25(OH)D. For HDL 0.002 (CI 0.048, 0.155)
European men (288)	60±11	3069	-TG +HDL	Age MVR p<0.05 for TG and HDL
US health care professionals Predominantly white (289)	63.8±8.6	900 All male	-TG +HDL	Age Linear trend across 25(OH)D quartiles; p for trend TG 0.001, for HDL <0.001
Danish adults (290)	30-60	4330 (48)	-TG -LDL-C	5 year follow up study. A 10mmol/L higher baseline 25(OH)D was associated with a drop in TG by 0.52% (p=0.03) and a drop in LDL-C by 0.66% (p=0.005).

Table 1.7 continued

Subjects (reference)	Age Years	N (% males)	Lipids (direction of association with 25(OH)D)	Adjustment Other information
Australian (AusDiab) (291)	Met S 52.3±12 No Met S 49.5±12.8	4164 (42.2)	-TG NS HDL-C	Age, gender, season, ethnicity, latitude, WC + Met S component at baseline. Prospective study of the associations of 25(OH)D with components of the Met S over 5 years P<0.01 for TG
British (292)	45 (range 41-46)	6810 (48)	-TG NS HDL	Age, gender, BMI Compared highest to lowest tertiles of 25(OH)D for risk of each Met S component OR for high TG 0.48 (CI 0.41-0.57) in the unadjusted model.

Key: NS=not significant; MVR=multivariate regression

1.9.5 25(OH)D and Blood Pressure

Observational studies have suggested higher blood pressures in winter months and at latitudes further from the equator, thus inferring that low 25(OH)D levels from reduced cutaneous synthesis is responsible for the observed differences in blood pressures (233). It is thought that the effect of 25(OH)D on blood pressure is mediated via its effect on the renin-angiotensin system (RAS). Laboratory studies have shown that VDR-null mutant mice have elevated levels of renin and angiotensin II leading to hypertension, cardiac hypertrophy and increased water intake (293). These abnormalities were prevented by treatment with an angiotensin converting enzyme (ACE) inhibitor. These findings were supported by the results of experiments that showed that 1,25$(OH)_2$D suppresses the expression of renin, suggesting that vitamin D may be a negative regulator of RAS (294).

Results of two observational studies are in keeping with the hypothesis that 25(OH)D exerts an effect on blood pressure (BP) via RAS. The LURIC study of 3316 subjects showed that in multivariate regression analysis both 25(OH)D and 1,25$(OH)_2$D were independent predictors of plasma renin and angiotensin (295). In a much smaller study, Vaidya et al noted that 25(OH)D was inversely associated with plasma renin activity in hypertensive subjects off medication (296).

Table 1.8 summarises the results of larger cross sectional studies on the association of 25(OH)D with blood pressure. Small studies have been included if they included Asian Indian or African subjects.

Table 1.8: Results of Cross Sectional Studies on the Association of 25(OH)D with Blood Pressure

Subjects (Reference)	Age (years)	N (% males)	Results (direction of association with 25(OH)D)	Adjustments Other information
NHANES US adults (297)	>20	12644 (48.2)	- SBP	BMI Quintiles of 25(OH) Significant inverse association between 25(OH)D and SBP.
British adults (292)	>45	6810 (48.4)	-BP	Age, gender and BMI Compared highest to lowest tertiles of 25(OH)D. OR for high BP 0.72 (CI 0.61-0.86) BP defined ≥140/90mmHg or use of antihypertensive medication
NHANES Caucasian and black adults (298)	20-49	7699 (47)	NS	Age A statistically significant inverse association between 25(OH)D and SBP was no longer significant when adjusted for age.
US adult health care workers Predominantly white (289)	63.8±8.6	900 (all male)	NS	Age Linear trend across quartiles of 25(OH)D. 31% had a history of hypertension in the lowest quartile compared to 25.4% in the highest quartile of 25(OH)D (p= 0.19).
US adults NHANES Three cycles (287)	≥20	3958 (48.2)	-SBP	Adjusted for several variables See Table 1.7 Quintiles of 25(OH)D. 25(OH)D inversely associated with SBP. Mean difference in BP per one standard deviation of 25(OH)D -0.032 (-0.062, -0.001)
Chinese adults (248)	50-70	3262 (44.2)	NS SBP -DBP	Adjusted for several variables See table 1.7 MVR DBP p=0.02
Korean adults	65.9	1330 (38.0)	-SBP NS DBP	Age, gender, BMI, season, PTH, calcium intake and serum calcium OR for SBP across quintiles of 25(OH)D, highest vs. lowest 0.47 (0.27, 0.82); p for trend= 0.007

Table 1.8 continued

Subjects (Reference)	Age (years)	N (% males)	Results (direction of association with 25(OH)D)	Adjustments Other information
Norwegian adults (299)	55-74	4125 (59.7)	-SBP -DBP	Age, gender, physical activity, BMI This was a cross sectional study carried out in 1994. In 2008 reassessed 25(OH)D and BP. 25(OH)D levels did not predict future hypertension. There was no significant association between change in 25(OH)D and change in BP
Australian adults (AusDiab)(291)	Met S 52.3± 12 No Met S 49.5 ± 12.8	4164 (42.2)	NS	Adjustments as for Table 1.7 OR for developing systolic or diastolic hypertension at 5 years per 25nmol decrease in baseline 25(OH)D 1.09 (0.95, 1.25)
Indian (253)	39.7 ±12.8	441 (53.7)	NS	No adjustments Sex stratified analysis showed no trend for increasing quintiles of 25(OH)D with any components of the Met S including BP
Urban South African black women (300)	57.6±9	291	-SBP NS DBP	Age, BMI, smoking, alcohol
US adults (255)	44-96	1070 (38.3)	NS	Age, season, physical activity,
Finnish smokers (301)	50-69	2271(all males)	-SBP	Negative association at baseline. Baseline 25(OH)D was not associated with SBP at four years.

Key: SBP=Systolic blood pressure; DBP=Diastolic blood pressure; NS=Not significant
MVR= multivariate regression

Effects of Intervention Studies on Serum Lipids and Blood Pressure

Several intervention studies have evaluated the effect of supplementation with vitamin D on components of the Met S. Jorde et al investigated the effects of supplementation with 25(OH)D over a one year period in Norwegian subjects (302). All subjects were either overweight or obese. Mean baseline 25(OH)D levels were 58±21.1nmol/L. This was a double blinded placebo controlled trial and subjects received either 40 000IU or 20 000IU 25(OH)D per week or placebo. All subjects received 500mg of calcium in addition. At end of the study, there were no significant differences in either blood glucose (by OGTT) or serum lipids between subjects and controls. Subjects who received 20 000IUI of vitamin D per week

showed a small but significant increase in systolic blood pressure (SBP) compared to the placebo group. The main aim of the study was to investigate the effect of vitamin D on weight change and it was therefore not powered to detect changes in glucose or lipid metabolism. Another year long randomized controlled trial of vitamin D supplementation (3332 IU/day) on healthy overweight subjects with mean 25(OH)D levels of 30 ±17.5nmol/L showed a significant decrease in serum TG and an increase in LDL-C compared to controls (281), but no effect on blood pressure. The mean age in this study was 48±10 years and baseline 25(OH)D levels were much lower than in the study by Jorde et al. On the other hand, in a three-year intervention trial in a group of post-menopausal women, vitamin D supplementation was shown to reduce HDL-C levels and increase serum LDL-C levels but did not change serum TG concentrations. Blood pressure changes were not evaluated (303).

The Women's Health initiative, designed to evaluate fracture and cancer risk in women receiving 400IU of vitamin D/day with either calcium or placebo, did not show any changes in either blood pressure or incident hypertension after seven years follow up (304).

There are very few studies that have been designed to evaluate blood pressure as a primary end point (305-307). The relatively small number of subjects and short duration of follow-up limit all these studies. Asian men failed to show any benefit from 120 000 IU of 25(OH)D supplementation fortnightly with regard to lipid profile, insulin sensitivity or blood pressure (308). The follow-up was only six weeks with 71 subjects. The subjects were all male with central obesity. Another of the few studies carried out on 89 Asian women and 84 Asian men who were severely 25(OH)D deficient, failed to show any change in lipid levels after supplementation with 400IU/day. Follow-up was for a year and was randomized and double blinded. Effects on blood pressure were not evaluated (309).

In a prospective double blinded trial aimed at determining the effect of various doses of supplementation with vitamin D_3 Forman et al noted that for each 2.5nmol/L increase in 25(OH)D there was a 0.2mmHg reduction in systolic blood pressure (SMP) ($p=0.02$) (307). They had 283 black participants from the US and the study was conducted over two years. It is debatable if racial differences modify the response to vitamin D therapy. Similar therapy with 4000 IU/daily of vitamin D_3 did not affect lipid profiles of South Asian women living in New Zealand

(310). Median baseline 25(OH)D levels of 25 nmol/L (11, 40) increased to 80nmol/L (67, 94) at the end of the six month period. Blood pressure changes were not assessed. Similarly, a number of meta-analyses have attempted to evaluate the effect of vitamin D supplementation on blood pressure but have also come up with inconclusive results (311-313).

To summarize, while the results of cross sectional studies suggest there is a negative association between 25(OH)D and serum TG, blood glucose and blood pressure, these have not been supported by data from RCTs. However, the majority of RCTs have been of very short duration and studies are often not powered to detect the effect of vitamin D supplementation on any of the components of the Met S. Short-term trials are probably inadequate to assess the effects of vitamin D supplementation on any outcomes because the optimal dose and duration of therapy is not known. Short-term supplementation does not maintain vitamin D sufficiency (177) and is therefore not suitable to detect long-term benefit or harm. Another reason for the lack of efficacy noted may be due to suboptimal dosaging of vitamin D. In his meta-analysis Pittas *et al* was able to detect a blood pressure lowering effect only if 1000 IU/day of vitamin D was used as opposed to <1000IU (313). Baseline 25(OH)D status influence outcomes, as Sugden showed that vitamin D therapy was beneficial in reducing blood pressure in those who were deficient to begin with (278). Finally, there ethnic specific differences possibly contribute to inconclusive results.

1.9.6 **25(OH)D and Obesity**

It has been suggested that obesity leads to lowered vitamin D status (314). The reasons for this are multifactorial, including the possibility that fat tissue acts as a reservoir for vitamin D_3 (315, 316). Obese individuals may have limited physical activity and hence less sun exposure (314, 317). However, the results of studies on this association are not always consistent, with some showing an inverse association between 25(OH)D and obesity (314, 318), whilst others have failed to show such an association (319-321). In a systematic review and meta-analysis of vitamin D in relation to BMI, Saneei et al showed a weakly significant inverse association between 25(OH)D and BMI in adults from developed countries, as well as in male adults in developing countries but not in females from developing countries (322). These studies were only three in number, all with small sample sizes and all from the Middle East (320, 321, 323). The results of RCTs are also conflicting with both beneficial effects of supplementation (318, 324) and lack of

benefit (281, 325) noted. Several factors confound these studies, for example the study size, use of calcium supplements which have been reported to cause weight loss in individuals with very low calcium intake (324), differences in 25(OH)D levels as well as ethnic differences.

Differences in body composition between males and females may potentially contribute to differences in 25(OH)D levels and the distribution of body fat differs by gender as well as ethnicity. A negative relationship between subcutaneous adipose tissue and visceral adipose tissue and serum 25(OH)D concentrations have been reported in Caucasians (317), Hispanics and African Americans (326), and South Asians (327). The relationship between 25(OH)D and body fat appears to be complex and has been shown to vary with age, race and gender (328). This has been attributed to the differential accumulation of 25(OH)D in the different adipose tissue depots. Again, the data on this is conflicting with some studies reporting that subcutaneous adipose tissue accumulates 25(OH)D following supplementation (329), while others suggest that visceral adiposity may explain ethnic specific differences (327).

1.10 SUMMARY AND RESEARCH GAPS

Vitamin D deficiency is common in many parts of the world, even in countries with abundant sunshine due to limited exposure to the ultraviolet light in the range of 290-315nm. This may be due to cultural practices around clothing or to limited time spent outdoors. Deficiency may also arise following the intake of very low calcium diets or conditions such as chronic kidney disease. There is a lack of data on the status of vitamin D in the various South African ethnic groups. A number of small studies have shown that there is seasonal variation in 25(OH)D levels and that levels are lower in urban dwellers compared with rural dwellers (92, 194).

Plasma concentrations of 25(OH)D of <30nmol/L are indicative of severe deficiency(330). Methodological differences in assays may contribute to large variation in measured levels. The evidence for the extra skeletal benefits of increased serum concentrations of 25(OH)D is limited, and we await the results of properly conducted large randomized controlled trials.
South Africa is a country that is undergoing a rapid transition with increasing urbanization of people and changing nutritional patterns. This has resulted in an epidemic of obesity and consequent increased cardiovascular risk (28, 331). It is

important to note that South Africa is an ethnically diverse country and results from other countries cannot be extrapolated to our unique population groups.

Vitamin D deficiency has also been reported to be associated with increased cardiovascular risk as shown by studies reporting an inverse association with a number of components of the Met S, i.e. blood glucose, serum lipids and blood pressure. Ethnic differences in body composition may contribute to the observed differences in vitamin D status.

BMD may be influenced both by body composition and by the vitamin D status of an individual. However, data on these associations is lacking in South African populations. In addition, data on the body composition of black South African males is lacking.

In addition, in a country that is experiencing an increasing burden of non-communicable diseases it is important to understand what the vitamin D status of our various population groups are, as well as what the predictors of deficiency may be and whether vitamin D deficiency is one of the drivers of this epidemic.

Hypothesis

The hypothesis to be tested in this thesis is that ethnic differences in vitamin D status contribute to ethnic differences in cardiovascular risk and BMD.

1.11 AIMS

The aims of this study are threefold:

1. Firstly, to determine the prevalence of vitamin D deficiency in a group of urban, healthy African and Indian populations resident in Johannesburg and the association of body composition with 25(OH)D levels

2. Secondly, to determine if ethnic differences in 25(OH)D contribute to differences in the prevalence of metabolic syndrome and its components

3. Thirdly, to compare the body composition of Black Africans and Indians and to determine if differences in body composition contribute to differences in vitamin D status, or to differences in BMD and whether ethnic differences in BMD are mediated by vitamin D levels.

CHAPTER 2

2. STUDY DESIGN AND METHODS

2.1 SUBJECT SELECTION

The study population consisted of two ethnic groups: Black African and Indian. Initial recruitment was done via the caregivers of the Birth to Twenty Cohort (Bt20). Bt20 is the largest and longest running longitudinal birth cohort study of child health development in Africa (332). Recruitment for the Bt20 study began in waves: at antenatal clinics, hospitals and clinics at time of delivery and at 'well-baby' services where immunisations were given during the first 18 months of the child's life. Enrolment took place between 23 April and 8 June 1990, and included singleton births of infants born in the Soweto-Johannesburg metropolis. A requirement was that the mothers resided within the metropolis for at least six months after the birth, so that infants who were born and registered onto the cohort but then moved out of the study area were later excluded and recorded as 'lost to follow up'. The realised sample was 3 273 families. All major South African ethnic groups were represented in the study but the majority were Black African, with Indian (4%), Whites (6%) and Mixed Ancestry (12%) making up the remainder.

A random number generator was used to select Black African and Indian caregivers of the cohort who were then contacted and asked if they had any members in their family that would be interested in participating in the study. The reason for not using the cohort subjects was that this study required subjects with an age range of 19-65 years. Subjects were predominantly residents of Soweto and Lenasia. Five hundred black families were contacted and all the Indian families of the Birth to twenty Cohort i.e. about 240. Additional Indian participants were recruited via the initial subjects. Recruitment was by ethnicity, age and gender. The aim was to have 60 subjects of each ethnicity and gender in three age group categories i.e. 19- <30, 30- <50 and 50-65 years (60 Black African males in each age group, and 60 Black African females in each age group and similarly for the Indian group). Exclusion criteria were age, (younger than18 years or above 65 years), pregnant or breast feeding, self-reported renal disease or eGFR <30 ml/min/1.73m^2, not of Black African or Indian ethnicity. The sample size was calculated to ensure a statistical power of 90% and a probability of less than 0.05 for detecting a 15% inter-group difference in 25(OH)D concentrations**.**Seven

hundred and thirty subjects were recruited. Two were excluded because they were of mixed ancestry and two others because of chronic kidney disease and liver disease respectively. One of the aims of the study was to determine the predictors of serum vitamin D levels which include supplementation with calcium and vitamin D. The details of the numbers included in each study are given in the relevant chapters. Written informed consent was obtained from each subject (Appendix 4). The Human Ethics Committee of the University of Witwatersrand approved the study protocol (ethics clearance number M10411- see Appendix 1).

2.2 BLOOD COLLECTION

Fasting blood samples were collected into plain gel separator vacutainer tubes (for serum), potassium fluoride tubes (for glucose) and EDTA tubes (for HbA1c and 25(OH)D). Samples were allowed to clot for half an hour and then centrifuged at 3000 rpm for 10 minutes. Serum and plasma was then aliquoted into 5ml plastic tubes. Samples were analysed the same day for all analytes except for 25(OH)D. The 25(OH)D samples were stored at -70^{0}C and analysed in batches of 60. All sample investigations were performed according to principles of good laboratory practice. All reagents used on the ADVIA 1800 and Centaur were supplied in kit form by Siemens Diagnostics. The ADVIA 1800 was used for measuring serum creatinine, calcium, phosphate, total cholesterol, HDL cholesterol, triglycerides, calcium, phosphate, albumin, plasma glucose, and glycated haemoglobin on whole blood, whilst the Centaur was used for measuring serum insulin, parathyroid hormone, and thyroid stimulating hormone.

2.3 BIOCHEMICAL METHODS

2.3.1 Determination of Serum Calcium

Test principle:

Calcium ions form a violet complex with 0-cresolpthalein complexone in an alkaline medium. The colour reaction is measured at 545/658nm.
Total coefficient of variation (CV) for the assay was 2.4% to 3.5%.
Reference Range: 2.15-2.55mol/L

2.3.2 Measurement of Serum Phosphate

Test principle:

Inorganic phosphate reacts with ammonium molybdate in the presence of sulphuric acid to form an unreduced phosphomolybdate complex which is measured at an endpoint reaction at 340/658nm.

$$\text{Phosphate + molybdate} \xrightarrow{H+} \text{phosphomolybdate complex}$$

Total CV 1.7% to 2.0%.

Reference Range: 0.78-1.42mmol/L

2.3.3 **Determination of Plasma Glucose**

Plasma glucose was analysed using a hexokinase method.

Test principle:

Under the action of hexokinase, D-glucose is phosphorylated with ATP to form glucose-6-phosphate (G-6-P). Glucose-6-phosphate is converted to 6-phosphogluoconate by glucose-6-phosphate-dehydrogenase (G-6-PDH) in the presence of NADP, which is converted to NADPH. The absorbance of NADPH is then measured in the UV region (340nm).

$$\text{Glucose + ATP} \xrightarrow{\text{Hexokinase}} \text{G-6-P + ADP}$$

$$\text{G-6-P + NADP+} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate + NADPH + H}$$

Total CV 4.9- 5.9 %

Reference Range: Fasting < 7mmol/L

2.3.4 **Determination of Serum Creatinine**

Test principle:

This is based on the reaction of creatinine with picric acid alkaline medium as described in the original reaction by Jaffe. A red coloured picrate-creatinine complex is produced. The rate of complex formation is measured at 505/ 571 nm and is proportional to the creatinine concentration. Rate blanking is used to minimize bilirubin interference. Because non-specific plasma proteins interactions give a positive bias of about 26.5µmol/L, 26.5µmol/L is subtracted from each result.

$$\text{Creatinine + picrate} \xrightarrow{OH^-} \text{Creatinine-picrate}$$

Total CV 6.1- 9.5%

Reference Range: Females 60-80 umol/L . Males 80-100 umol/L

2.3.5 **Estimated GFR (eGFR)**

Estimated glomerular filtration rate was calculated using the Modification of Diet in Renal Disease (MDRD) equation as described by van Deventer et al (333). This equation is given below:

eGFR $^{=}$ 175 x [S-Cr (umol/L)/88.4]$^{-1.154}$ x age (years) $^{-0.203}$ x (0.742 if female)

Reported as ml/min/1.73m^2

Where S-Cr = serum creatinine CKD staging dependent of eGFR

2.3.6 **Determination of Glycated Haemoglobin (HbA1c)**

Glycated haemoglobin (HbA1c) is formed by the non-enzymatic glycation of the N-terminus of the β-chain of hemoglobin A. The level of HbA1c is proportional to the level of glucose in the blood and is widely accepted as an indicator of the mean daily blood glucose concentration over the preceding two months. Glycated haemoglobin is described as a percentage of the total haemoglobin (tHb) content. To determine the tHb concentration spectrophotometrically, all different forms of hemoglobin must be converted into one single form with a uniform absorption spectrum. The concentration of HbA1c and the concentration of tHb are measured and the ratio is reported as percentage HbA1c. In a pre-treatment step, the whole blood sample is mixed with Haemoglobin Denaturant Reagent ((Siemens Diagnostics, Tarrytown, USA (1:41dilution)) and incubated for a minimum of 5 minutes at room temperature. The red blood cells are lysed and the hemoglobin chain is hydrolyzed by the protease present in the reagent.

For the measurement of total hemoglobin, Total Hemoglobin Reagent (Siemens Diagnostics, Tarrytown, USA) is used. The method is based on the conversion of all hemoglobin derivatives into alkaline hematin in an alkaline solution of a nonionic detergent as described in the original procedure of Wolf, Lang, and Zander (334).

A latex agglutination inhibition method is used for the measurement of specific HbA1c. HbA1c present in the sample competes with the agglutinator (synthetic latex containing multiple copies of the immunoreactive portion of HbA1c) for the anti-HbA1c antibody; thereby reducing the rate of agglutination. A concentration curve is obtained by monitoring the change in scattered light as a change of

absorbance. The actual change in absorbance is inversely proportional to the concentration of HbA1c in the sample. The percentage HbA1c is calculated using the HbA1c and tHb values.

Total CV 1.8% to 2.0%.

Good control <7%

2.3.7 **Determination of Serum Cholesterol**

Test principle:

This is an enzymatic colorimetric test. Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{cholesterol} + \text{RCOOH}$$

Cholesterol is converted by oxygen with the aid of cholesterol oxidase to cholest-4-ene-3-one and hydrogen peroxide.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholest-4-ene-3-one} + H_2O2$$

The hydrogen peroxide created forms a red colour by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The colour intensity is directly proportional to the concentration of cholesterol and the absorbance of the complex is measured at an end point reaction at 505/694nm.

$$2H_2O_2 + 4\text{ aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 2H_2O$$

Total CV 2.0% to 2.2%

Desirable ranges depend on cardiovascular risk factors.

Category 1 Risk <4.5mol/L

2.3.8 **Determination of High-Density Lipoprotein Cholesterol (HDL-C) in Serum**

Test principle:

This method consists of two distinct reaction steps:

The elimination of chylomicrons, VLDL-cholesterol and LDL cholesterol by cholesterol esterase and cholesterol oxidase. The peroxide produced is released by catalase.

Cholesterol esterase

Cholesterol Ester ⟶ Cholesterol + RCOOH

Cholesterol + O_2 $\xrightarrow{\text{Cholesterol oxidase}}$ Cholestenone + H_2O_2

This is followed by the measurement of HDL-C after release of HDL-C by surfactant. The intensity of the quinoneimine dye produced in the Trinder reaction is directly proportional to the HDL-C concentration when measured at 596nm.

Cholesterol Ester ⟶ Cholesterol + RCOOH

Cholesterol + O_2 ⟶ Cholestenone + H2O2

H_2O_2 + 4-aminoantipyrine + HDAOS $\xrightarrow{\text{Peroxidase}}$ Quinoneimine + $4H_2O_2$

HDAOS=N (2-hydroxy-3-sulfopropyl)-3, 5-dimethoxyaniline

Total CV 2.1% to 2.6%.

Desirable ranges depend on cardiovascular risk factors.

Category 1 Risk Males >1.0mmol/L. females >1.2 mmol/L

2.3.9 **Determination of Serum Triglycerides**

Test principle:

The triglyceride method is based on the Fossati 3 step enzymatic reaction based with a Trinder endpoint (335, 336). This method quantitates total triglycerides including mono- and diglycerides and the free glycerol fractions. The triglycerides are converted to glycerol and free fatty acids by lipase. Glycerol is converted to glycerol-3-phosphate by glycerol kinase followed by its conversion by glycerol-3-phosphate oxidase to hydrogen peroxide. A coloured complex is formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. The absorbance of the complex is measured at an endpoint reaction at 505/694nm.

Triglycerides + 3H2O $\xrightarrow{\text{Lipase}}$ Glycerol + Fatty Acids

ATP + Glycerol $\xrightarrow{\text{Glycerol Kinase}}$ Glycerol-3-phosphate + ADP

O2 + Glycerol-3-phosphate $\xrightarrow{\text{Glycerol-3-phosphate oxidase}}$ $2H_2O_2$ +

Dihydroxyacetone phosphate

Peroxidase

$2H_2O_2$ + 4-Aminophenazone + 4-Chlorophenol ⟶ Quinoneimine dye + H_2O

Total CV 0.9% to 2.4%

Desirable ranges depends of cardiovascular risk factors .

Category 1 risk <1.7 mmol/L

2.3.10 **Calculation of Low-Density Lipoprotein Cholesterol (LDL-C)**

The conventional method of ultra-centrifugal estimation of cholesterol is time consuming and expensive. For this reason LDL-C is most commonly estimated from measurements of total cholesterol, HDL-C and plasma triglycerides using the Friedewald equation (as below) when all analytes are measured in mmol/L(337).

LDL-C = (Total cholesterol) - (HDL-C) - (TG/2.2).

HDL-C=High density lipoprotein cholesterol

TG=triglycerides

Importantly, the Friedewald equation should not be used when chylomicrons are present, when serum triglycerides exceed 4.5mmol/L, and from patients with dysbetalipoproteinemia.

Desirable range depends on cardiovascular risk factors.

Category 1 risk <2.5 mmol/L

2.3.11 **Determination of Plasma Intact Parathyroid Hormone (PTH)**

Test principle:

This is a two-site sandwich immunoassay using chemiluminescent technology, which uses constant amount of two anti-human PTH antibodies. The first antibody is a polyclonal anti-human PTH antibody to the N-terminal (1-34) region labelled with acridinium ester. The second antibody is a biotinylated polyclonal goat anti-human PTH antibody to the 39-84 region. Stretptavidin in the solid phase is covalently linked to paramagnetic latex particles. A direct relationship exists between the amount of PTH present in the patient sample and the amount of relative light units detected by the system.

Total CV 3.4 -5.2%

Reference Range: 1.2-8.5 pmol/L

2.3.12 **Determination of Serum Insulin**

Test principle:

This is a two-site sandwich immunoassay that uses a constant amount of two antibodies. The first antibody is a monoclonal mouse anti-insulin antibody labelled

with acridinium ester. The second antibody is also a monoclonal mouse anti-insulin antibody covalently coupled to paramagnetic particles. A direct relationship exists between the amount of insulin present in the patient sample and the amount of relative light units detected by the system.

Total CV 6.3% to 7.5%.

Reference Range: 3-25 mU/L

2.3.13 **Determination of Thyroid Stimulating Hormone (TSH)**

Test principle:

This is a two-site sandwich immunoassay using chemiluminescent technology using constant amounts of two antibodies. The first antibody is a monoclonal mouse anti-TSH antibody labelled with acridinium ester and the second is a polyclonal sheep anti-TSH antibody. A direct relationship exists between the amount of TSH present in the patient sample and the amount of relative light units detected by the system.

Total CV 3.17 to 5.87%

Reference Range: 0.35-4.5 mIU/L

2.3.14 **Determination of Plasma 25(OH)D**

Plasma 25(OH)D was measured using the Clin Rep high performance liquid chromatography (HPLC) kit (Recipe, Munchen, Germany). In this analytical method, 25(OH)D was determined from plasma using HPLC with a photodiode array (PDA) detector. Prior to HPLC analysis a short liquid-liquid extraction was performed. The liquid-liquid extraction includes a precipitation step that denatured the protein bond and extracted the 25(OH)D out of the lipid layer into a clear upper layer. Fifty microliter of the eluant was injected onto the HPLC system where the analytes were separated on the appropriate C18 analytical column (Recipe, Munchen Germany). The separated $25(OH)D_2$ and $25(OH)D_3$ were detected at a wavelength of 264nm. The concentration was calculated using a standard curve with two sets of controls run every 20 samples. The standards and controls were human based lyophilized samples that are traceable to the NIST 25(OH)D standard. The chromatograms were integrated using peak height. Total vitamin D (25(OH)D) was taken as the sum of $25(OH)D_2$ and $25(OH)D_3$. The intra-assay and inter–assay coefficient of variation (CV) for $25(OH)D_3$ for controls at a mean of 61.7 nmol/L ranged from 0.36-9.4%, and for controls at a mean of 222 nmol/L ranged from 2.1-5.5%. For $25(OH)D_2$ the CV for the low control (mean 49.2

nmol/L) was 6.8-9.7% and for the high control (mean 199 nmol/L) ranged from 1.1-5.7%. The limit of quantification was 6.5 nmol/L for $25(OH)D_3$ and 11.0 nmol/L for $25(OH)D_2$. Any $25(OH)D_2$ below the limit if quantification was assigned a nominal value of zero.

Reference Range: <12.5 nmol/L severe vitamin D deficiency

2.4 ANTHROPOMETRIC MEASUREMENTS

2.4.1 Weight, Height and Waist Circumference

Weight (digital scale, Dismed, USA) to the nearest 100g, and height (stadiometer, Holtaine, UK) to the nearest millimetre were measured with subjects wearing light clothing and no shoes. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters (kg/m^2). A BMI of $20kg/m^2$ to $24.9kg/m^2$ is defined as normal, $25kg/m^2$ to $29.9kg/m^2$ defined as overweight and $\geq 30kg/m^2$ as obese (338).These cut off points were derived primarily in European populations to correspond to risk thresholds for a wide range of chronic diseases and mortality.

The waist and hip circumferences were measured with the subjects standing, feet together and arms at the sides. Waist circumference was measured with a soft measuring tape to the nearest 0.5cm at the level of the smallest girth above the umbilicus in the standing position and hip circumference was measured at the widest part of the buttocks (Appendix 5)

2.4.2 Body Composition Analysis

Dual Energy X-ray Absorptiometry (DXA)

Body composition of the total body was determined by dual energy X-ray absorptiometry (DXA; Hologic, Bedford, MA, USA (computer software version 12.5:7)) according to standard procedures of the International Society of Clinical Densitometry. Scans were conducted using the automatic scan mode, i.e. 'array', 'fast array' or 'slow array', depending on the weight of the subject. Subjects wore light clothing having removed metal objects, jewellery, etc. DXA was used to measure bone mineral content (BMC, in grams), bone area (BA, in square centimetres), and areal bone mineral density (BMD in grams per centimetre) of whole body, total hip, femoral neck, and lumbar spine. The coefficients of variation (CV %) for repeated measurements of the manufacturers phantom were 0.3, 0.4

and 0.2% for BA, BMC and BMD, respectively. The CV for repeated measurement by the DXA operator of the lumbar spine and total hip were 0.7 and 1.0% respectively. DXA scans for whole body were analysed less head as many women wore wigs and hair weaves that could not be removed prior to scanning. This artificial hair was of similar density to soft tissue and could cause measurement artefact. Total body fat and lean body mass (in grams) were also measured by DXA.

Ultrasound Analysis of Visceral and Subcutaneous Adiposity

Abdominal visceral (VAT) and subcutaneous adiposity (SCAT) was determined by ultrasonography using a LOGIC ultrasound system (USS) (GE Healthcare, Piscataway, NJ) with a 2-5MHz 3C-RS curved array transducer. Ultrasound VAT thickness was defined as the distance measured in cm from the peritoneum to the vertebral bodies. Ultrasound SCAT thickness was defined as the depth measured in cm from the skin to the linea alba. The scan depth was set at 15cm for the visceral fat measure and 9cm for the subcutaneous fat measure to visualize the relevant anatomical structures. Both measurements were obtained at the site where the xyphoid line and waist circumference met. A single trained operator took all measurements.

This technique has been validated for the measurement of visceral and subcutaneous adiposity (339)

CV <2%. This was calculated after the same operator took duplicate measurements on 15 subjects.

2.5 QUESTIONAIRRES

2.5.1 Food Frequency Questionnaire

A seven-day recall food frequency questionnaire was used to assess nutrient intake (Appendix 2). A trained worker conducted interviews in English. The food intake questionnaire used in our study was validated for the South African population. Nutrient analyses of calcium intake were calculated only from the consumption of dairy products, and for Vitamin D from intake of fish, eggs and margarine. Portion sizes were reported in household measures and then converted to weights using standard tables (340) . Food intake in grams was calculated for seven days to be able to include quantities of less than one gram per day in the analysis. Thereafter, the nutrient content was calculated and expressed as average nutrients consumed per day for each subject. Nutrient

analyses were carried out using the following computer software: Food Finder III Nutritional Intervention Research Unit and Research Information Systems Division, Medical Research Council, South Africa.

2.5.2 Sunshine Exposure Score

Sunshine exposure was determined using a questionnaire, which assessed the participants' recollection of daily sun exposure over the previous week (341). There were three options for time spent outdoors every day (0 = ≤ 5minutes, 1 = 5 - 30minutes and 2 = > 30minutes). There were four options for skin exposure while outdoors (1 = face and hands only, 2 = face, hands and arms, 3 = face, hands, and legs and 4 = bathing suit). A daily sun exposure score (min = 0; max = 8) was calculated by taking the product of the amount of time spent outdoors and the amount of skin exposed. The seven days' sun exposure scores were then summed to give the weekly sun exposure score (min = 0; max = 56).

Seasons of the year, for the statistical analysis, were categorized as winter (June to August), spring (September to November), summer (December to February) and autumn (March to May).
(Appendix 3)

2.5.3 Smoking Status, Educational Status and Alcohol Intake

A standardized questionnaire was used to collect information on age, sex, visit date, smoking (current, former, never), self-reported diabetes, hypertension, dyslipidaemia, coronary heart disease and medication use. Alcohol intake was not quantified but was categorized as yes or no.
(Appendix 3)

2.5.4 Blood Pressure

Blood pressure was measured thrice in the right arm, with the subject seated and after 5 minutes rest using an automated sphygmomanometer. The final reading was used (Appendix 5).

2.5.5. Statistical Analysis

Statistical methods are given in the methods section of each chapter.

CHAPTER 3

3. THE EFFECT OF ADIPOSITY, SEASON, DIET AND SUPPLEMENTATION ON VITAMIN D STATUS OF HEALTHY URBAN BLACK AFRICAN AND INDIAN ADULTS

ABSTRACT

Vitamin D deficiency has been implicated in the aetiology of infectious diseases and metabolic syndrome. These are prevalent in the Black African and Indian populations within South Africa; however, there are limited data on 25(OH)D concentrations in these populations.

The aim of this study was to assess the vitamin D status and its predictors in healthy adults in Johannesburg.

We assessed the vitamin D status of 714 adult Black African and Indian subjects resident in Johannesburg. The contributions of sunshine exposure, season, dietary intake of calcium and vitamin D supplements, total body fat and body fat distribution to 25-hydroxyvitamin D (25(OH)D) concentrations were assessed. 25(OH)D was measured by high performance liquid chromatography (HPLC). The contribution of $25(OH)D_3$ to total 25(OH)D was assessed.

The mean age of the population was 42.6 ± 13.1 years (range: 18-65). 28.6% of Indians had 25(OH)D concentrations < 30 nmol/L in comparison to 5.1% in the Black African group ($p<0.0001$). Parathyroid hormone (PTH) was negatively associated with 25(OH)D while season and sunshine exposure were positive predictors explaining 16% of the variance ($p<0.0001$) in Black Africans. In Indians, PTH was negatively associated with 25(OH)D while male gender, season and calcium supplementation were positive predictors and explained 17% of the variance of 25(OH)D ($P<0.0001$). In multivariate regression analysis, neither total body fat nor body fat distribution was predictive of 25(OH)D in either group.

Local conditions such as sun exposure, use of dietary supplements, and ethnicity are important determinants of plasma 25(OH)D concentrations.

3.1 INTRODUCTION

South Africa like the rest of sub-Saharan Africa is plagued by the burden of chronic infectious diseases including tuberculosis (TB) and HIV (342) as well as a rising burden of non-communicable diseases (343). Recently studies have shown that vitamin D deficiency or insufficiency is associated with an increased risk of cardiovascular disease (289) diabetes (344), cancers (345, 346), infections (92) and autoimmune conditions (347) in addition to its classical effects on bone.

Although its definition remains a concern, several studies on vitamin D deficiency across the world suggest that it is a global health problem (13, 348). South African studies have been limited to children (192), or women (194), or hospitalized or ill subjects only (92) and the reported prevalence of vitamin D deficiency ranges from 8.0% in Black African children to 62.7% in adult subjects with TB and/or HIV, using a cut-off of <50 nmol/L to define deficiency.

In order to understand the health risks associated with vitamin D deficiency it is important to know the determinants of 25(OH)D concentrations. The objective of our study was to determine the vitamin D status in Black African and Indian groups in Johannesburg and to identify the main determinants of 25(OH)D concentrations in each ethnic group, including body fat mass and body fat distribution. Factors like sunshine exposure, age, fat mass, gender, ethnicity and lifestyle may influence the serum concentrations of this vitamin (13, 327, 349, 350) and may be important contributors to vitamin D status. The serum level of 25(OH)D has been reported to be inversely associated with body mass index (BMI) and with subcutaneous as well as visceral adiposity (317, 330). We also determined the contributions of $25(OH)D_3$ and $25(OH)D_2$ to total 25(OH)D using HPLC as several commonly used immunoassays preferentially detect $25(OH)D_3$ (351).

3.2 METHODS

The methods used in this chapter have been described in detail in chapter two. They include the measurement of serum calcium, phosphate, creatinine for estimation of GFR, plasma parathyroid hormone and 25(OH)D, measurement of weight, height and waist circumference, total body fat by DXA and visceral and subcutaneous adiposity by ultrasonography. Intake of calcium and vitamin D was determined, supplementation with calcium and vitamin D, educational status and smoking status. Seven hundred and fourteen subjects for whom we had complete

data were included in this study, 371 Black African and 343 Indian (340 males and 374 females).

3.2.1 **Statistical Analysis**

Analysis was conducted using the Stata software package version 12 (College Station, TX, USA). The Shapiro-Wilk's test was used to test for normality. The results of non-normally distributed data are reported as median and interquartile range (IQR) and for normally distributed data as mean and SD. Non-normally distributed data was transformed to achieve normality (log transformed or square root) before regression analysis. The Mann-Witney U test was used to compare differences between the two ethnic groups for non-normally distributed data, the Student t-test for normally distributed data and the chi-squared test for categorical data. A p value of <0.05 was considered statistically significant. We compared differences in 25(OH)D between the different ethnic groups by ANOVA. Pair-wise comparisons were used only if the overall ANOVA was statistically significant. Bonferroni corrections for multiple comparisons were used.

The main aim of this study was to identify the principal determinants of 25(OH)D concentrations in each ethnic group and therefore data for Black Africans and Indians were analysed separately. Regression analysis was restricted to total 25(OH)D, rather than to $25(OH)D_2$ and $25(OH)D_3$ individually. We carried out univariate Spearman's analysis to assess the strength of the relationship of 25(OH)D with parameters that are thought to influence 25(OH)D status. The following were considered as potential predictors based on previous evidence: age (88), season (352), body fat (353), sunshine exposure (340) and supplementation with vitamin D or calcium (354), renal function, smoking status(169), dietary intake of calcium and vitamin D (355) and PTH concentrations. These as well as any remaining study variables, mostly anthropometric measures, that gave correlations of $p<0.2$ in univariate analysis were used as independent variables in separate multivariable models for Black Africans and Indians in which 25(OH)D was the dependent variable. Values were reported as standardized β coefficients. In each ethnic group the season with the lowest 25(OH)D concentrations were coded as 1 and the remainder were grouped together and coded as 2. In the Black African subjects, winter was coded as 1 and in the Indian subjects, spring.
Backward stepwise multiple regression analysis was performed until only variables with a p value of <0.05 remained. We adjusted all models for gender and height. Due to collinearity, BMI and total body fat were not included in the same model.

We also developed two models, one which included individual fat measures together with other variables mentioned above in case of collinearity and then a second with all fat measures entered together. We tested for collinearity of models that included visceral adiposity, subcutaneous adiposity and total body fat and there was no collinearity as evidenced by a variance inflation factor of <10.0. We also tested for interaction between ethnicity and body composition variables and noted that there was no significant interaction. To facilitate direct comparisons of the strengths of the associations, the results of the regression models are reported as standardised β values.

3.3 RESULTS

3.3.1 Demographic Variables

The demographic and biochemical characteristics of the participants are described in Table 3.1.

The mean age of the Black African subjects and Indian subjects were similar, 41.6 ± 13.1 years (range 19-65) and 43.5 ± 13.0 years (range 18-65; p=0.06), respectively. More Indian than Black African subjects had completed high school (p<0.0001) and there were more smokers amongst the Black African than among the Indian subjects (34% vs. 17%, p<0.0001). None of the participants were on anti-epileptic medication, but 12.9% of Indians and 1.4% of Black Africans were on statin therapy. HIV status was determined by self-report: 87.7% of Black Africans reported their status as negative, 8.5% reported being HIV positive and 3.7% had never been tested; 94.5% of Indians reported their status as negative while 5.5% had never been tested. To our knowledge none of the participants suffered from metabolic conditions likely to impact vitamin D metabolism.

Table 3.1: Descriptive Characteristics of Study Population

Variable	Black African (n=371)	Indian (n=343)	P value
Age (years)	41.6 ± 13.1	43.5 ± 12.9	0.06
Males (N (%))	180 (48)	163 (45)	0.65
Education: high school not complete (N (%))	209 (57.0)	114 (33.0)	<0.0001
Smoker (N (%))	124 (33.8)	58.0 (16.6)	<0.0001
BMI (kg/m^2)	26.2 (21.7, 31.7)	26.7 (23.3, 31.0)	0.38
Waist circumference (cm)	89.0 (78.0, 102)	95.0 (85, 106)	<0.0001
Visceral adipose thickness (cm)	4.93 (3.72, 6.34)	4.91 (3.31 ,6.46)	0.48
Subcutaneous adipose thickness (cm)	2.32 (1.59, 3.44)	3.04 (2.24, 3.91)	<0.0001
Total body fat (kg)	21.5 (11.9, 31.6)	24.3 (18.1, 32.6)	<0.0001
eGFR ($ml/min/1.73m^2$)	87.9 (77.7,103)	88.1 (75.7,103)	0.90
$25(OH)D_3$(nmol/L) **25(OH)D(nmol/L)** **$25(OH)D_3$ females (nmol/L)#** **25(OH)D females (nmol/L)** **$25(OH)D_3$ males (nmol/L)#** **25(OH)D males (nmol/L)**	62.8 (45.5, 88.5) 64.9 (46.4, 89.4) 56.8 (40.4, 80.1)* 58.3 (42.9, 85.6)* 72.4 (51.1, 94.1) 73.2 (51.2, 94.1)	36.7 (24.7, 53.4) 41.2 (28.4, 56.8) 32.4 (19.0, 48.9)* 35.8 (23.0, 54.5)* 43.9 (30.6, 58.9) 45.4 (33.6, 62.7)	<0.0001 <0.0001 <0.0001 <0.0001 <0.0001 <0.0001
Ca (mmol/L)	2.27 ± 0.09	2.26 ± 0.11	0.21
Pi (mmol/L)	1.10 ± 0.17	1.10 ± 0.15	0.30
PTH (pmol/L)	4.70 (3.30, 6.50)	4.85 (3.61, 6.92)	0.09
Ca intake diet (mg/day)	223 (98.6, 427)	336 (204, 487)	<0.0001
Vit D intake diet (ug/day)	2.96 (1.50, 5.12)	1.17 (0.55, 2.11)	<0.0001
Vit D deficient (N (%))	22.0 (5.90)	106 (30.3)	<0.0001
Weekly sunshine score	17.8 ± 10.7	16.0 ± 9.53	0.23
Ca supplements (N (%)	12.0 (3.24)	77.0 (22.5)	<0.0001
Vit D supplements (N (%)	7.00 (1.89)	51.0 (14.9)	<0.0001

Key: Data given as mean ± SD, median (IQR) or N (%). 25(OH)D deficiency defined as <30nmol/L, insufficiency as 30.0-49.9 nmol/L. PTH=parathyroid hormone, ALP=alkaline phosphatase, eGFR=estimated glomerular filtration rate, Pi =inorganic phosphate. *P<0.0005 for females versus males of same ethnic group. #For Black African females $ P <0.0001 for Black African males vs Indian males @ p<0.0001 for Black African females vs Indian females n=191, for Indian females n=180, for Black African males n=180 and for Indian males n=163

3.3.2 Anthropometry

Median BMI was in the overweight range for both groups. Indian subjects had greater waist circumference, greater total body fat and greater percentage body fat compared to the Black African group ($p<0.0001$ for all variables). Visceral adiposity was similar between both ethnic groups but Indian subjects had greater subcutaneous adiposity than the Black African subjects (Table 3.1) did.

3.3.3 Vitamin D Status

Using the Shapiro-Wilk's test, 25(OH)D was not normally distributed. Black African subjects had significantly higher 25(OH)D and $25(OH)D_3$ when compared to the Indian population (see Table 3.1). Gender differences were noted for 25(OH)D as well as $25(OH)D_3$. The prevalence of vitamin D deficiency (25(OH)D level of <30nmol/L) was 28.6% in Indians compared to just over 5% in Black Africans (Table 3.1) and was highest in Indian women (37.9% in Indian females and 17.9% in Indian men, $p <0.0001$; 6.7% in Black African females and 2.8% in Black African males, $p=0.08$).

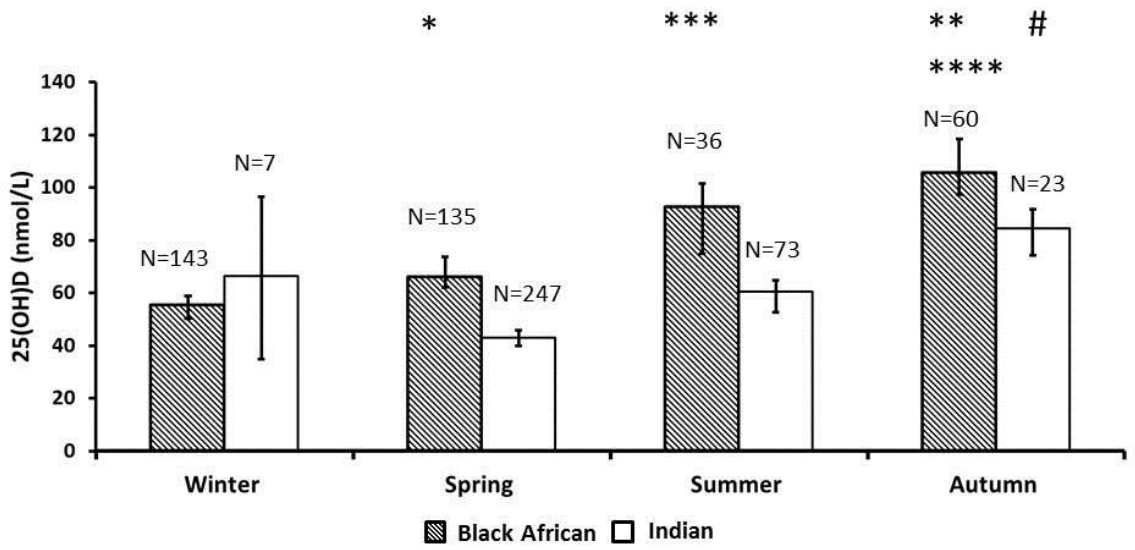
Across all groups, 94.5 ± 17.9% of all 25(OH)D measured was $25(OH)D_3$. Mean serum calcium and phosphate concentrations were normal and similar across ethnicities. Plasma PTH concentrations were raised above the laboratory reference range in 12% of Africans and 14% of Indians.

Seasonal Differences

As expected, seasonal differences were noted in 25(OH)D concentrations in both groups. The plasma 25(OH)D level was 40 to 60% higher in subjects in whom blood was collected in autumn compared to those who had samples taken in winter/spring (Figure 1). This variation was due to changes in $25(OH)D_3$ with all participants showing maximal $25(OH)D_3$ concentrations in autumn. As expected, no significant seasonal difference was observed for $25(OH)D_2$ concentrations. The prevalence of 25(OH)D deficiency (<30 nmol/L) per season was as follows: 8% in winter, 23% in spring, 13% in summer and less than 1% in autumn. The prevalence of insufficiency (25(OH)D < 50 nmol/L) per season was 40% in winter, 31% in spring, 30% in summer and 4% in autumn.

Sunshine exposure score was not significantly different between the ethnic groups but was different by gender: Black African male, 18.80 ± 8.49, Black African

female, 16.73 ± 11.33 (p=0.02); Indian male, 18.71 ± 10.19, Indian females, 14.43 ± 8.46 (p=0.0001).



Black Africans: * P = 0.005 spring vs. winter, **P < 0.05 autumn vs. summer, ***P = 0.001 summer vs. winter, **** P < 0.001 autumn vs. winter and spring
Indians: # P <0.001 autumn vs. spring and summer
(Adjusted for gender using ANCOVA)

Figure 3.1: Seasonal Variation in 25(OH)D According to Ethnicity

Supplementation and Medication: Indian Population

Among the Indian population, 22% were on calcium supplementation and 15% were on vitamin D supplementation (Table 3.1). The 25(OH)D concentrations were higher in those Indian participants on vitamin D supplementation (52.2 (32.7, 73.2) nmol/L versus 39.2 (26.9, 55.1) nmol/L; p<0.001) or calcium supplementation (54.2 (35.8, 80.4) nmol/L versus 37.5 (26.3, 53.8) nmol/L; p<0.0001).

25(OH)D concentrations were not significantly different between Indian males on statins (49.3 (30.3, 60.9) nmol/L) compared to those not on statins (45.1 (34.0, 60.9) nmol/L; p=0.7) but were higher in Indian females on statins (52.2 (30.1, 80.8) nmol/L) compared to those not taking statins (34.4 (22.4, 52.9) nmol/L; p=0.04).

Supplementation and Medication: Black African Population

3% of Black African subjects were on calcium supplementation and 2% on vitamin D supplementation (p<0.0001 for both comparisons with Indian group) (Table 3.1).

Dietary Intake of Calcium and Vitamin D

Dietary calcium intake was significantly lower in Black African (223 (98.6, 427) mg/day) compared to Indian subjects (336 (204, 487) mg/day; p<0.0001). Dietary vitamin D intake was higher in Black African (2.95 (1.55, 5.12) µg/day) than Indian subjects (1.17 (0.50, 2.11) µg/day; p<0.0001). 7.5% Black Africans and 1.2% Indians had a dietary intake of vitamin D ≥ 10ug/ day (p=0.008). Similarly, 4.5% of Black Africans had daily calcium of ≥ 1000mg/day and 2.6% of Indian had a daily calcium intake of ≥ 1000mg/day (p=0.002).

3.3.4 Univariate Analysis for Associations with 25(OH)D

The correlations of 25(OH)D with other variables are shown in Table 3.2. Univariate analyses showed that serum PTH was negatively correlated with 25(OH)D in both ethnic groups and weekly sunshine score was significantly positively associated with 25(OH)D in Black Africans but not in Indians. In terms of body composition, visceral and subcutaneous adiposity as well as total body fat were negatively associated with 25(OH)D in the Black African group. In the Indian population, none of the body composition variables were significantly associated with 25(OH)D. There was no significant association of 25(OH)D status with age, calcium or vitamin D intake in either group.

Table 3.2: Spearman's Correlation of 25(OH)D with Other Variables

Variable	25(OH)D Black Africans (n=371)	25(OH)D Indians (n=343)
Age (years)	0.002 (0.96)	0.03 (0.54)
PTH(pmol/L)	-0.26 (<0.0001)	-0.23 (<0.0001)
Calcium intake (mg/day)	0.03(0.55)	0.08(0.14)
Vitamin D intake (ug/day)	-0.008(0.88)	0.07(0.17)
BMI (kg/m^2)	-0.09 (0.09)	-0.02 (0.67)
Visceral adipose thickness (cm)	-0.18 (0.0007)	0.07 (0.19)
Subcut adipose thickness (cm)	-0.12 (0.02)	-0.07 (0.17)
Whole body fat (kg)	-0.11 (0.04)	-0.07 (0.20)
Weekly sunshine score	0.23 (<0.0001)	0.02 (0.67)

Values given are spearman's coefficient, ρ (p) PTH=parathyroid hormone, BMI=body mass index

3.3.5 Predictors of 25(OH)D Concentrations

To assess the predictors of 25(OH)D concentrations in more detail, we performed multivariable regression analyses (see Table 3.3). In Black Africans (see model 1), season during which the blood sample was taken, PTH, and weekly sunshine score explained 16% of the variance for 25(OH)D. Season and weekly sunshine score were positive predictors of 25(OH)D concentrations whilst PTH was a negatively related to 25(OH)D.

In the Indian subjects' season of blood sample collection, PTH, gender and supplementation with calcium explained 17% of the variance of 25(OH)D (see Table 3.3, model 2). Gender, season of blood sample collection and the use of calcium supplements all being positive predictors.

Table 3.3: Multivariable Regression Models for 25(OH)D

Regression Model No.	Ethnic Group	Dependent Variable (nmol/)	Independent Variables with Beta Coefficient (p value)		R^2 for Full Model (P-value)
1	Black African (n=371)	25(OH)D nmol/L	Season PTH (pmol/L) Weekly sunshine	0.26 (<0.0001) -0.21 (<0.0001) 0.10 (0.05)	0.16 (<0.0001)
2	Asian-Indian (n=343)	25(OH)D nmol/L	Season PTH (pmol/L) Calcium supplementation Gender	0.12 (0.02) -0.17 (0.001) 0.30 (<0.0001) 0.21 (<0.0001)	0.17 (<0.0001)

Key: R^2 are unadjusted values. Values given are standardised beta coefficients (p-value). The following were log transformed: PTH and 25 (OH) D. Season: Winter was the reference season for Africans and spring the reference season for Indian subjects (see the Statistical Analysis sub-section of the Methods section for a full description of the coding). Female gender was coded as 0 and male as 1

3.4 DISCUSSION

To our knowledge, this is the first study to examine determinants of 25(OH)D concentrations in Black African and Indian subjects living in Africa. As noted by Prentice et al., Africa is a heterogeneous continent with differences in latitude, climate, food availability, skin pigmentation and cultural practices (356). A systematic review of global vitamin D status by Wahl et al (348) noted that there were lack of data from many African countries. They noted mean concentrations between 25 and 49 nmol/L in a single South African study (357) with higher concentrations noted in West and Central Africa (348). Hilger et al (2014) noted

similar findings in a more recent systematic review of vitamin D status in populations worldwide (358). We have shown that Indians living in Johannesburg have a high prevalence of 25(OH)D deficiency (28.6%), while deficiency is rare in Africans (5%) when using a cut off <30 nmol/L to define deficiency.

Comparison across studies is complicated by the different cut off levels used to define vitamin D deficiency and insufficiency and by variations in assay methodology. However, our findings of increased prevalence of vitamin D insufficiency in the Indian population are in keeping with a number of studies (13, 180, 359). Mithal et al noted that 25(OH)D concentrations below 75 nmol/L are prevalent in every region of the world while concentrations < 25 nmol/L are most common in South Asians and the Middle East (13).
Ethnicity has been consistently related to circulating 25(OH)D concentrations in adults (360, 361) and a high prevalence of hypovitaminosis D has been reported from Indian studies carried out in India as well (178, 180, 359) as well as from Indian subjects in the United Kingdom (362). The differences noted in our study for 25(OH)D concentrations between the two ethnic groups probably reflect differences in diet, clothing, and exposure to sunshine.

Mean 25(OH)D concentrations in our Black African population was higher than mean 25(OH)D level from Cape Town (357). This may be because of differences in latitude, age of the subjects as well as assays used to measure 25(OH)D. Concentrations were also higher than those noted in non-Hispanic blacks and Mexican-Americans from the US [(297)] but much lower than concentrations noted in indigenous populations in East Africa (363). It appears that urban Black Africans have lower 25(OH)D concentrations compared to rural subjects in the older age groups (>50 years) (194).

In spite of the fact that Johannesburg experiences between eight and nine hours of sunshine a day throughout the year, we noted clear seasonal variation of 25(OH)D in both ethnic groups with season a significant positive predictor in both Indians and in Black Africans. The 25(OH)D concentrations were lowest for collections in the months of July to September (late winter, spring) indicating that sunshine exposure does influence 25(OH)D concentrations in both communities. Clear seasonal changes in 25(OH)D status similar to our findings have previously been reported in South Africa from several different groups of subjects such as the elderly with fractures (352) in HIV positive and negative subjects from Cape Town

(92) as well as in healthy Black African and Caucasian children living in Johannesburg(192). The sunshine exposure score was a predictor of 25(OH)D in our Black African population but not in the Indian population, amongst whom females were mostly veiled. Using univariate regression analysis weekly sunshine exposure was a significant predictor of 25(OH)D in the Indian population. However, after adjusting for age, and gender and season, weekly sunshine exposure, was no longer a significant predictor of 25(OH)D concentrations in the Indian population. The sunshine exposure score comprised time spent outdoors as well as the extent of clothing coverage. It is possible that time of day, and character of time spent outdoors (e.g. physical activity) are also important in determining UV exposure and these were not assessed in this study. Combined these findings suggest that seasonal variation in 25(OH)D concentrations observed in Johannesburg among Black African and Indian subjects is probably a result of both the time spent outdoors and the amount of clothing worn.

There was no significant association of 25(OH)D status with age in either group; while male gender was a predictor of higher 25(OH)D concentrations in the Indian population. This study was not able to determine the reasons for this difference but it is likely to be related to cultural practices such as wearing of the veil.

We could find only one other study which has examined the relationship between body fat distribution and 25(OH)D in Indians (327) and our findings are in contrast to theirs. They showed a negative association between visceral fat and 25(OH). When the fat measures were all included together the same model none of the fat measures were predictive of 25(OH)D. The reasons for this difference are not known. Because of possible collinearity a second regression model with total body fat, visceral and subcutaneous fat included individually was developed and neither total body fat nor body fat distribution was predictive of 25(OH)D in either group (data not presented).

There are major differences between the two studies: the Indian population in Johannesburg are quite a homogenous population all having migrated from India whereas the cohort described by Sulistyoningrum et al (327) originated in India, Pakistan, Bangladesh, Sri Lanka and Nepal. Visceral adiposity and serum vitamin D concentrations were each measured in the two studies by different techniques; the sample size in the current study (343 versus 192) was higher. The mechanisms by which circulating concentrations of 25(OH)D are influenced by adipose tissue mass (or vice versa) are not fully understood. However, it is known

that adipose tissue acts as a large depot for vitamin D (364) which may in turn significantly affect 25(OH)D bioavailability (316). Furthermore, obese individuals display a reduced response of serum 25(OH)D concentrations to UV-B radiation (316) and oral vitamin D supplementation (365) when compared with subjects who are not obese. It is interesting to note that data from the National Health and Nutrition Examination Survey III (NHANES III) suggest that the relationship between body fat and 25(OH)D is stronger in white than in black subjects (366).

Calcium supplementation was positively predictive of 25(OH)D in Indians, while vitamin D supplementation was not. This discrepancy may be because many calcium supplements contain vitamin D, but the amount was not specifically quantified in our study. Significantly more Indian participants were on calcium and vitamin D supplementation than were Black African participants. We postulate that this may be due to differences in educational and socio-economic status. Dietary intake of vitamin D was low because vitamin D content in most foods is naturally low and our estimates of vitamin D intake did not include the contribution from supplements. Consequently, the lack of a relationship between dietary vitamin D intake and serum 25(OH)D is not surprising and supports sunshine exposure as the major determinant of vitamin D status in these populations. In our study, dietary calcium intakes were much lower in both ethnic groups than the 1000 mg/day intake recommended by the National Osteoporosis Foundation of South Africa. Low calcium intake could result in poor vitamin D status because of increased 25(OH)D metabolism. Despite the low dietary intake of calcium and vitamin D, the plasma concentration of 25(OH)D was much higher in the Black African compared to the Indian population. This highlights the important contribution of sunshine exposure to 25(OH)D concentrations in this part of the world.

PTH concentrations were not significantly different between the ethnic groups, despite a high proportion of Indian subjects with vitamin D deficiency. Attempts have been made to define optimal vitamin D status for bone health based on the relationship between PTH and 25(OH)D (367) and this relationship appears to vary by race (148, 368). Thus, in African-Americans PTH concentrations are maximally suppressed at lower 25(OH)D concentrations compared with whites (148). The current study shows that the significantly lower 25(OH)D concentrations in the Indian compared to the Black African subjects occurs in the presence of similar PTH concentrations. This data further suggests that the relationship between

25(OH)D and PTH varies by race and that in terms of bone health optimal 25(OH)D concentrations may be different in each of these two population groups.

Our model explained between 16-17% of the variance in 25(OH)D concentrations which is comparable to data from previous studies (369-371). Thus, McDonnell et al analysed data on non-food factors affecting vitamin D status and showed that these explained only 13% of the variance in total 25(OH)D concentrations (370). Similarly, analysis of potential determinants of 25(OH)D in the US Radiologists Technologists Study showed that despite detailed data on UV radiation exposure only 25% of the variance in 25(OH)D concentrations was explained (371). Other factors that contribute to variance include skin colour (372), physical activity (371) and genetic factors (112), none of which were assessed in this study.

Visceral adiposity is strongly associated with cardiometabolic diseases (373). In South Africa the mortality from cardiovascular disease and the prevalence of type 2 diabetes is higher in Indian compared with Black African subjects (374). However, the current study demonstrates that the level of visceral adipose tissue is similar in these two population groups. This suggests that factors other than visceral adiposity are important contributors to cardiovascular risk in the Indian population.

The strengths of this study include the large sample size, the method used to measure plasma 25(OH)D, the multi-ethnic nature of the study population, the determination of total body fat and body fat distribution and the assessment of a large number of variables that are thought to influence vitamin D status. There are however a number of limitations of this study which include the cross sectional nature of the study which allows us to draw inferences as to the determinants but does not permit definitive determination of the causative nature of any associations. Secondly, we did not quantify supplementation with vitamin D or calcium and could therefore not accurately assess the contribution of these supplements to 25(OH)D concentrations. Furthermore, dietary intake of calcium and vitamin D was limited to their quantification in fish, eggs and dairy products, but not meat. We also did not assess physical activity that might influence the dermal synthesis of vitamin D and visceral and subcutaneous adiposity were determined by ultrasound and not by CT scan, which is the gold standard method. However, this method has been validated in this population (339). The questionnaire used to assess sunshine exposure has been used in a number of

studies (341, 375) but has not been validated against personal UV dosimetry or observed exposure. The limit of quantitation for 25(OH)D_2 was 11 nmol/L and because of this 25(OH)D_2 was not quantifiable in 9.2% of participants. 25(OH)D_2 only constitutes a small proportion of total 25(OH)D in South Black African populations, thus its lack of detectability in some subjects had little influence on the overall vitamin D status of the two ethnic groups. Finally, the number of subjects studied in each season was not evenly distributed throughout the year.

3.5 CONCLUSIONS

This study clearly demonstrates that any policy on vitamin D supplementation should be considered in a local context. Thus, season and by implication differences in weather conditions, use of dietary supplements and ethnicity are all strong determinants of plasma 25(OH)D concentration and these factors, which vary widely across nations, must be considered before deciding on supplementation programs. Adequate vitamin D concentrations in different population groups also require further study. In our study, almost a third of the Indians who participated in this study were vitamin D deficient. It is possible that this group will benefit from routine supplementation. In view of the seasonal changes noted further consideration should be given to whether year long supplementation is required or if supplementation can be restricted to winter and early spring. Studies are required to determine optimal dosages. The use of calcium was associated with higher 25(OH)D concentrations in Indians, but the benefits and its impact on cardiovascular health have to be assessed (353, 376). In conclusion, our study has shown that there are major differences in 25(OH)D concentrations in apparently healthy adults living in Johannesburg, with Indians showing the highest prevalence of 25(OH)D deficiency. Important positive predictors of 25(OH)D in this population are supplementation and season.

As we have shown that there are clear differences in 25(OH)D status between Indian and Black African groups the next study aimed to determine if these differences contribute to ethnic differences in the prevalence of Met S and its individual components.

CHAPTER 4

4. THE ASSOCIATION OF 25 HYDROXYVITAMIN D AND PARATHYROID HORMONE WITH METABOLIC SYNDROME IN TWO ETHNIC GROUPS IN SOUTH AFRICA

ABSTRACT

Though inconsistent, a number of studies have shown an association between vitamin D (25(OH)D) status, parathyroid hormone (PTH) and metabolic syndrome (Met S). These have largely been carried out in Caucasians or black subjects living in high income countries. There are no data on the relationship of 25(OH)D and PTH status with Met S in populations resident in Africa.

The aims of this study were to evaluate if there was an association of 25(OH)D or PTH with Met S in non-Caucasian populations in South Africa, and whether these molecules explained ethnic differences in the prevalence of Met S and its individual components.

We measured anthropometry, serum 25(OH)D and PTH levels and the components of Met S, plus related metabolic variables, in 374 Black African and 350 Indian healthy adults from the greater Johannesburg metropolitan area.

Metabolic Syndrome was diagnosed in 29% of the Black African and 46% of the Indian subjects ($p<0.0001$). Subjects with Met S had higher PTH than those without Met S, ($p<0.0001$), whilst 25(OH)D levels were not significantly different ($p=0.5$). In multivariate analysis, 25(OH)D was not associated with any components of Met S however, PTH was shown to be positively associated with systolic ($p=0.018$) and diastolic ($p=0.005$) blood pressures and waist circumference ($p<0.0001$), and negatively associated with Homeostasis Model Assessment ($p=0.0008$) levels. Logistic regression analysis showed that Indian ethnicity (OR 2.24; 95% CIs 1.57, 3.18; $p<0.0001$) and raised PTH (OR 2.48; 95% CIs 1.01, 6.08; $p=0.04$) adjusted for 25(OH)D produced an increased risk of Met S but 25(OH)D did not (OR 1.25; 95% CIs 0.67, 2.24; $p=0.48$).

Plasma PTH but not 25(OH)D is an independent predictor of Met S in Black African and Indians in South Africa.

4.1 INTRODUCTION

The incidence of obesity is on the rise in many low and middle income countries (LMICs) (377) and South Africa is no exception, with a national survey showing that 60% of South African women were either overweight or obese (9). Obesity is associated with an increased risk of cardiovascular disease (CVD) and additional risk factors include hyperglycaemia or type 2 diabetes (T2DM), hypertriglyceridemia, low high-density lipoprotein cholesterol (HDL-C), and hypertension. These CVD risk factors are commonly found in subjects with abdominal obesity and this grouping together of CVD risk factors has been termed metabolic syndrome (Met S) (22).

The burden of disease related to CVD is expected to increase substantially in LMIC, such as South Africa, over the next decades and it is important to understand traditional and non-traditional risk factors that contribute to this disease burden. Some populations are more susceptible to CVD because of specific factors e.g. familial hypercholesterolaemia in the South African Afrikaner population (378) or insulin resistance in the Indian population (379). The prevalence of cardiovascular and diabetes risk factors is higher in Indians than in Black African population groups in South Africa despite greater obesity in the latter population (380).

Several studies in various parts of the world suggest that vitamin D deficiency or insufficiency may increase the risk of Met S and its sequelae; T2DM and CVD.(40, 41) Vitamin D is a hormone precursor which before exerting its metabolic effects undergoes two successive hydroxylation steps. The first converts vitamin D to 25 hydroxyvitamin D (25(OH)D) and the second converts 25(OH)D to the main active hormonal form 1,25 dihydroxy vitamin D ($1,25(OH)_2D$). Hypovitaminosis D appears to exert its effects via reductions in intracellular calcium and through the effects of $1,25(OH)_2D$ on the regulation of various target genes e.g. by lack of suppression of the renin gene leading to hypertension (381) and reduced insulin secretion through reduction in islet β –cell function (41).

Elevated PTH levels have also emerged as a potential risk factor for Met S (288, 382). Parathyroid Hormone has been shown to be inversely associated with insulin sensitivity (260) and directly with blood pressure (383, 384). Both 25(OH)D and PTH are calciotrophic hormones and their metabolism is inversely related such that a decrease in serum 25(OH)D leads to a rise in PTH (385). It is currently

unclear whether the association noted between hypovitaminosis D and components of Met S is mediated by 25(OH)D or PTH or by both.

There are no studies from Africa that have investigated any associations between 25(OH)D, PTH and components of Met S. Therefore, the aims of this study were to examine the association of 25(OH)D and PTH with Met S in Black African and Indian population groups in South Africa and to determine whether ethnic differences in 25(OH)D or PTH levels contribute to differences in the prevalence of Met S, its component parts and related metabolic variables within these populations. The hypotheses to be tested were that 25(OH)D is lower and PTH is higher in Met S patients in both population groups, and that ethnic differences in the prevalence of Met S and its component parts are related to ethnic differences in the blood level of 25(OH)D.

4.2 METHODS

Details of subject recruitment, biochemistry and anthropometry are described in chapter two. The following were measured: weight, height and waist circumference, blood pressure, serum glucose, creatinine, insulin, total cholesterol, triglycerides and HDL cholesterol, plasma PTH and 25(OH)D. Seven hundred and seventeen subjects were included in this study for whom we had complete data, 373 Black Africans and 344 Indians (342 males and 375 females).

4.2.1 Diagnosis of Metabolic Syndrome, and Measurement of Insulin Resistance and Glomerular Filtration Rate

Met S was determined using the harmonized definition (22) Thus, subjects had to have at least three of the following criteria: increased waist circumference (≥94cm for Black African males, ≥80cm for Black African or Indian females, ≥90cm for AI males); elevated serum triglycerides (≥1.7mmol/L); reduced serum HDL-C (<1mmol/L for males and 1.3 mmol/L for females); elevated blood pressure (systolic blood pressure ≥130mmHg and /or diastolic pressure ≥85mmHg) and elevated fasting blood glucose (≥5.6mmol/L). Insulin resistance was measured using the HOMA technique (386) and estimated glomerular filtration rate (eGFR) was calculated by The Modified Diet in Renal Disease formula (MDRD) (333).

4.2.2 **Statistical Methods**

Stata 12 (College Station, TX, USA) was used for all analyses. The distribution of variables was assessed by the Shapiro-Wilk's W test and by examination of the normal probability plot. Logarithmic transformations were applied for all non-normally distributed variables. In descriptive analysis, normally distributed, continuous variables were reported as mean ± Standard Deviation (SD) whilst non-normally distributed data were expressed as median (interquartile range (IQR)). Categorical data were reported as N (percent).

Pearson's correlation was used to assess the association of 25(OH)D and PTH with the selected variables. Multivariable regression analyses were conducted to assess the association of Met S components with 25(OH)D and PTH after adjusting for potential confounders i.e. age, gender and body mass index (BMI). Ethnicity was also included as an independent variable to determine if ethnic differences in Met S components were mediated by 25(OH)D or PTH.

In order to determine if the higher risk of Met S in the Indian population was mediated by 25(OH)D or PTH, logistic regression analysis was performed with the stepwise addition of 25(OH)D and PTH to a model that also included ethnicity, age, gender and BMI as independent variables. The attenuation of the p-value (from significant to non-significant) for the ethnicity odds ratio was used as the indicator of any effect.

PTH was found to increase the risk of Met S and therefore we analysed which of the individual components of Met S were influenced by PTH by separately adding each individual component to a logistic regression model for Met S risk which included PTH, age, gender, ethnicity, BMI and 25(OH)D as independent variables. As described above, the attenuation of the p-value, in this case for the PTH odds ratio, was used as the effect indicator.

In all regression models, Africans were coded as 0 and Indians as 1; whilst females were coded as 0 and males as 1.

4.3 RESULTS

4.3.1 Ethnic Differences

Six participants were excluded from the analyses due to incomplete data collection and seven participants with serum calcium above the normal reference range (2.15-2. mmol/L) were also excluded. Characteristics of the participants (n=717) are shown in Table 4.1. Both ethnic groups had similar ($p>0.05$) age, BMI, HDL-C, eGFR, calcium and PTH levels. Indian subjects had significantly higher fasting glucose, triglycerides and total cholesterol ($p<0.0001$ for each of the 3 comparisons) compared to the Black African population. They also had a greater waist circumference ($p=0.0001$) and were more insulin resistant ($p<0.0001$). Black African subjects had higher 25(OH)D levels and systolic and diastolic blood pressures ($p<0.0001$ for all three comparisons).

When these differences were analysed by gender Indian males had significantly higher cholesterol, triglycerides, fasting glucose and waist circumference compared to Black African males ($p<0.0001$ for all) whilst Black African males had higher systolic blood pressures ($p<0.0001$) HDL-C ($p <0.005$) and eGFR ($p<0.05$).

Indian females also had significantly higher cholesterol, triglycerides, fasting glucose (<p<0.0001) and eGFR ($p<0.005$) compared with Black African females. Both Indian males and Indian females were more insulin resistant than their Black African counterparts ($p<0.0001$)

Table 4.1: Characteristics of Subjects According to Ethnicity and Gender

Variable	Black African	Indian	p-values	Black African Males	Black African Females	p-values	Indian Males	Indian Females	p-values
N	373	344	-	181	192	-	161	183	-
Age (years)	41.6 ± 13.1	43.5 ± 12.9	0.06	42.0 ± 13.2	42.0 ± 13.1	0.9	42.0 ± 13.2	46.0 ± 12.7	0.5
Glucose (mmol/L)	4.90 (4.60, 5.00)	5.21 (4.80, 5.70)	<0.0001	4.90# (4.60, 5.40)	4.90 * (4.60, 5.30)	0.5	5.20 (4.91, 5.80)	5.10 (4.80, 5.63)	0.03
Triglycerides (mmol/L)	0.85 (0.62, 1.27)	1.26 (0.83, 1.85)	<0.000.1	0.93# (0.67, 1.42)	0.82* (0.57, 1.17)	0.03	1.37 (1.00, 2.08)	1.10 (0.77, 1.60)	<0.0001
HDL-C (mmol/L)	1.28 (1.08, 1.53)	1.26 (1.07, 1.53)	0.81	1.20## (1.01, 1.43)	1.33** (1.17, 1.59)	<0.0001	1.12 (1.00, 1.26)	1.45 (1.24, 1.73)	<0.0001
Systolic blood pressure (mmHg)	131 (120, 146)	123 (113, 134)	<0.0001.	133# (121, 148)	129* (119, 143)	0.16	125 (118, 135)	118 (108, 132)	0.0003
Diastolic blood pressure (mmHg)	83.0 (76.0, 93.0)	80.0 (73.1, 87.0)	<0.0001	82.0 (74.0, 93.0)	83.0* (77.3, 93.2)	0.40	82.0 (75.3, 89.1)	78.2 (72.0, 85.2)	0.006
Waist (cm)	89.0 (78.0, 102)	95.0 (85.0, 106)	0.0001	85.0# (75.0, 99.0)	95.0 (82.0, 105)	<0.0001	98.0 (90.5, 108)	93.0 (82.0, 103)	0.0006
eGFR (ml/min/1.73m^2)	87.9 (77.7,103)	88.1 (75.7,103)	0.9	88.7### (74.5,103)	87.4** (77.4,103)	0.92	84.2(72.7,96.3)	92.8 (81.6,108)	<0.0001
Calcium (mmol/L)	2.27 ± 0.09	2.26 ± 0.11	0.21	2.28 ± 0.08	2.27 ± 0.10	0.32	2.26 ± 0.1	2.26 ± 0.1	0.63
Cholesterol (mmol/L)	4.22 ± 1.04	4.97 ± 1.10	<0.0001	3.93# ± 0.99	4.24*± 1.07	0.002	4.72 ± 1.17	4.97 ± 1.04	0.16
BMI (kg/m^2)	26.2 (21.7, 31.7)	26.7 (23.3, 31.0)	0.38	23.3 (20.2, 27.3)	29.9 (24.3, 35.3)	<0.0001.00	26.2 (23.7, 30.4)	27.1 (23.0, 32.8)	0.36
HOMA	1.83 (1.10, 2.90)	3.23 (2.13, 5.01)	<0.0001.00	1.43 (0.80, 2.69)	2.16 (1.31, 2.92)	0.0001	3.45 (2.15, 5.50)	3.15 (2.05, 4.60)	0.12
PTH (pmol/L)	4.70 (3.30, 6.50)	4.85 (3.61, 6.92)	0.09	4.30 (3.00, 5.60)	5.30*** (3.80, 5.90)	0.0002	4.60 (3.60, 6.40)	5.00 (3.70, 7.20)	0.09
25(OH)D (nmol/L)	70.9 (51.5, 95.1)	41.8 (28.6, 56.8)	<0.0001	72.7 (51.1, 94.1)	58.3 (42.9, 85.6)	0.0008	46.8 (33.6, 62.7)	35.7 (23.0, 54.5)	0.0002

Data given as mean ± SD for normally distributed data and median (IQR) for non-normally distributed data. Black African Males vs Indian males, # p<0.0001, ## p<0.005, ###p<0.05, Black African females vs Indian females *p<0.0001, ** p<0.005, ***P, 0.05. PTH=parathyroid hormone, HOMA= homeostatic model of assessment, BMI=body mass index, eGFR= estimated GFR

The month of the year during which blood samples were drawn for 25(OH)D measurement may be a confounding variable since 25(OH)D levels are known to vary across the seasons (352). However, ethnic differences in plasma 25(OH)D levels were still observed irrespective of the season during which the blood sample was obtained. Vitamin D levels were highest for both ethnic groups during the autumn months (data not shown).

4.3.2 **Gender Differences**

In both ethnic groups females had lower 25(OH)D levels compared to their male counterparts ($p=0.0008$ in Black Africans and $p=0.0002$ in Indians) and this was also observed for triglyceride levels ($p=0.03$ in Black Africans and $p<0.0001$ in Indians) (Table 4.1). The PTH levels were higher in Black African females than in males ($p=0.0002$), and although the same pattern was observed in Indians, the gender effect was not significant ($p=0.09$). No gender differences were observed for age or calcium levels in either ethnic group. In Black African females, cholesterol ($p=0.002$), BMI ($p<0.0001$) and HOMA ($p=0.0001$) were higher than in males, but no gender effect for these variables was seen in the Indian population. Indian females had higher eGFR than Indian males ($p<0.0001$) but no gender difference was observed in the Black African cohort. In Indian males, glucose ($p=0.03$) and systolic ($p=0.0003$) and diastolic ($p=0.006$) blood pressure were higher than in females but no differences were noted between sexes in the Black African subjects. Within both ethnic groups, HDL-C levels were higher in females than in males ($p<0.0001$ for both). Waist circumference was significantly higher in Black African females than in males ($p<0.0001$), but in the Indian subjects this trend was reversed ($p=0.0006$).

4.3.3 **Met S**

Met S was diagnosed in 38% of the participants, with a prevalence of 29% in the Black Africans and 46% in the Indian group ($p<0.0001$). The data in Table 2 show that within each ethnic group the most prevalent Met S component was increased waist circumference (94.5% in Black African and 96.9% in Indians; $p=0.33$) and elevated triglyceride was the least common (36.7% in Black Africans and 54.7% in Indians; $p=0.004$). The occurrence of raised fasting glucose levels was more common in Indians than in Black African Met S patients ($p=0.009$), whilst raised systolic blood pressure was more common in Black African than in Indian Met S subjects ($p=0.005$). Low HDL-C ($p=0.28$) and raised diastolic blood pressure ($p=0.13$) were not significantly different in frequency across the two ethnic groups

(Table 4.2). HOMA-IR was greater n Indian females compared to Black African females ($p<0.0001$) and in Indian males compared to Black African males ($p<0.0001$) (data not shown).

Table 4.2: Prevalence of Individual Components of Met S in Each Ethnic Group

Variable	BA with Met S (N=109)	I with Met S (N=161)	p-values
Raised glucose	48.0 (44.0)	97.0 (60.3)	0.009
Raised triglycerides	40.0 (36.7)	88.0 (54.7)	0.004
Reduced HDL-C	74.0 (67.9)	91.0 (61.5)	0.28
Raised systolic blood pressure	96.0 (88.9)	121 (75.2)	0.005
Raised diastolic blood pressure	83.0 (76.9)	110 (68.3)	0.13
Raised waist circumference	103 (94.5)	156 (96.9)	0.33

Data given as N (%) BA - African I - Indian

In both ethnic groups, subjects with Met S had higher ages, HBA1c, HOMA, PTH, cholesterol and BMI measures than those without Met S, whilst eGFR was lower in those with Met S ($p<0.05$ for all comparisons) (see Table 4.3). Calcium ($p=0.80$) and 25(OH)D levels ($p=0.50$) were not significantly different across these 2 groups (Table 4.3).

Table 4.3: Comparison of Subjects with and Without Met S within Each Ethnic Group

	BA without Met S	BA with Met S	p-values	AI without Met S	AI with Met S	p-values
N	260	109	-	186	158	-
Age (years)	39.1 ± 12.9	47.7 ± 11.3	<0.0001	38.4 ± 12.7	49.4 ± 10.5	<0.0001
HBA1c (%)	5.31 (5.06, 5.32)	5.64 (5.35, 6.36)	<0.0001	5.33 (5.04, 5.56)	5.76 (5.41, 6.13)	<0.0001
HOMA	1.44 (0.87, 2.35)	2.85 (2.00, 4.00)	<0.0001	2.43 (1.72, 3.58)	4.56 (2.99, 6.77)	<0.0001
PTH (pmol/L)	4.50 (3.25, 5.95)	5.41 (3.92, 7.60)	0.0002	4.40 (3.50, 6.20)	5.40 (4.13, 7.62)	0.0001
25(OH)D (nmol/L)	72.3 (52.6, 95.4)	67.4 (49.9, 94.8)	0.45	48.3 (34.8, 66.0)	50.5 (34.7, 70.1)	0.50
eGFR ml/min/1.73m^2	91.3 ± 23.0	84.2 ± 19.3	<0.0001	93.1 ± 20.2	87.2 ± 19.1	0.003
Calcium (mmol/L)	2.27 ± 0.09	2.28 ± 0.09	0.30	2.26 ± 1.11	2.27 ± 0.10	0.80
Cholesterol (mmol/L)	4.02 ± 0.95	4.48 ± 1.05	0.001	4.86 ± 1.03	5.11 ± 1.17	0.04
BMI (kg/m^2)	24.0 (20.0, 29.0)	30.9 (27.4, 35.1)	<0.0001	24.9 (21.1, 29.6)	28.8 (25.3, 32.6)	<0.0001

Data given as mean ± SD for normally distributed data and median (IQR) for non-normally distributed data BA=African AI=Indian. BMI=body mass index, eGFR= estimated GFR, PTH= parathyroid hormone

4.3.4 Univariate Analysis

In a univariate analysis there was a significant negative correlation between PTH and 25(OH) D ($p<0.0001$, see Table 4.4) and a significant trend for PTH to be higher in Indian subjects ($p=0.04$) and in females ($p=0.0002$).

Table 4.4: Pearson's Correlations for PTH and 25(OH)D for all Subjects

Variable	Log PTH	Log 25(OH)D
Age	0.26 (<0.0001)	-0.02 (0.52)
Log BMI (kg/m^2)	0.22 (0.0001)	-0.03 (0.38)
eGFR ($ml/min/1.73m^2$)	-0.07 (0.05)	-0.11 (0.003)
Log Glucose (mmol/L)	0.09 (0.02)	-0.06 (0.09)
Cholesterol (mmol/l)	0.11 (0.005)	-0.17 (<0.0001)
Log Triglyceride (mmol/L)	0.14 (<0.0001)	-0.08 (0.02)
HDL-C (mmol/L)	0.009 (0.81)	-0.06 (0.08)
Log Systolic blood pressure (mmHg)	0.15 (0.0001)	0.12 (0.001)
Log Diastolic blood pressure (mmHg)	0.19 (0.0001)	0.08 (0.02)
Log HOMA	0.09 (0.02)	-0.02 (0.67)
Log PTH (pmol/L)	-	-0.19 (<0.0001)
Log 25(OH)D (nmol/L)	-0.24 (<0.0001)	-
Log Waist (cm)	0.25 (<0.0001)	0.0002 (0.99)

Data given as r (p-value) eGFR =estimated GFR,

We observed significant ($p<0.05$) positive associations of PTH with age, BMI, glucose, HOMA, triglyceride, cholesterol, waist circumference and systolic and diastolic blood pressures. The 25(OH)D levels correlated negatively with BMI, and GFR and positively with systolic and diastolic blood pressure ($p<0.05$ for all associations). There were significant trends for 25(OH)D to be higher in Black African and in male subjects ($p<0.001$ for all comparisons).

4.3.5 Multivariable Regression Analysis

Multivariate associations of 25(OH)D and PTH with components of Met S are presented in Table 4.5, with age, gender and BMI included as independent variables in all models.

Table 4.5: Multiple Regression Models for Determining the Effects of Ethnicity, PTH and 25(OH)D on Components of Met S

Dependent Variable	MODEL NUMBERS AND INDEPENDENT VARIABLES					
	MODEL 1	MODEL 2		MODEL 3		
	Ethnicity	Ethnicity	25(OH)D (log)	Ethnicity	25(OH)D (log)	PTH (log)
Log Glucose (mmol/L)	0.02 (0.013)	0.02 (0.015)	-0.003 (0.47)	0.02 (0.015)	-0.003 (0.44)	-0.01 (0.43)
Log Triglycerides (mmol/l)	0.13 (<0.0001)	0.13 (<0.0001)	-0.001 (0.89)	0.13 (<0.0001)	0.0007 (0.94)	0.04 (0.32)
Log HDL-C (mmol/L)	0.0002 (0.99)	-0.0007 (0.93)	-0.007 (0.11)	-0.0009 (0.93)	-0.006 (0.175)	-0.005 (0.78)
Log Systolic blood pressure (mmHg)	-0.04 (<0.0001)	-0.04 (<0.0001)	0.002 (0.34)	-0.04 (<0.0001)	0.002 (0.28)	0.02 (0.018)
Log Diastolic blood pressure (mmHg)	-0.02 (<0.0001)	-0.02 (<0.0001)	0.003 (0.14)	-0.02 (<0.0001)	0.004 (0.11)	0.03 (0.005)
Log Waist (m)	0.02 (0.002)	0.02 (0.001)	0.003 (0.37)	0.02 (0.005)	0.004 (0.19)	0.09 (<0.0001)
Log HOMA	0.23 (<0.0001)	0.23 (<0.0001)	0.0009 (0.93)	0.23 (<0.0001)	-0.0003 (0.97)	-0.13 (0.008)

Data given as beta coefficient (p-value); all models adjusted for age, gender and BMI. Ethnicity, 25(OH) D and PTH were added in a forward, stepwise manner.

Addition of ethnicity to all the models demonstrated that Indian ethnicity was a positive determinant of glucose, triglyceride and HOMA levels but a negative determinant of diastolic and systolic blood pressure. Addition of 25(OH)D to these models had minimal effect on the beta values for ethnicity and in none of the models was 25(OH)D shown to be a determinant of any of the components of Met S, or HOMA. When PTH was added to each of the models, neither the ethnicity or 25(OH)D beta values were affected, however PTH was shown to be positively and significantly associated with systolic ($p=0.018$) and diastolic ($p=0.005$) blood pressures and waist circumference ($p<0.0001$) and negatively associated with HOMA ($p=0.008$) levels. Addition of PTH/25(OH)D ratio did not change results.

Logistic regression was used to determine whether the higher prevalence of Met S in the Indian population was related to PTH or 25(OH)D levels. The data in Table 4.6 show that after adjusting for age, gender and BMI, Indian ethnicity carries a 2.24 (95% CIs 1.57, 3.18; $p<0.0001$) increased risk of Met S compared to Black African subjects (see Table 4.6, model 1).

Table 4.6: Multivariate Logistic Regression Analysis for Determining the Effect of Ethnicity, PTH and 25(OH)D on Risk of Met S

Model Numbers	Independent Variables	Odds Ratios	95% CIs	p-values
1	Ethnicity	2.24	1.57, 3.18	<0.0001
2	Ethnicity Log 25(OH)D	2.29 1.15	1.60, 3.28 0.78, 1.70	<0.0001 0.47
3	Ethnicity Log 25(OH)D Log PTH	2.27 1.25 2.48	1.56, 3.30 0.67, 2.24 1.01, 6.08	<0.0001 0.48 0.04

All models adjusted for age, gender and BMI, with Met S as the dependent variable. Ethnicity, 25(OH)D(mmol/L) and PTH(pmol/L) were added in a forward, stepwise manner.

Additional adjustment for 25(OH)D had no effect on the p-value (or odds ratio (OR)) for ethnicity and had no significant effect itself (see Table 4.6, model 2). The final addition of PTH to the logistic regression model (see Table 4.6, model 3) had no effect on the p-value or OR for ethnicity or 25(OH)D, but did have a significant effect itself (OR=2.48; 95% CIs 1.01, 6.08; p=0.04).

In order to determine which individual components of Met S are influenced by PTH to increase the risk of Met S, logistic regression analysis was performed. The results in Table 4.7 show that the increased risk for Met S conferred by PTH arises principally through its effect on systolic blood pressure and its positive association with waist circumference. This is demonstrated by the fact that adding these variables individually to the logistic regression model attenuates the p-value of the OR for PTH (see Table 4.7, models 6 and 7).

Table 4.7: Risk of Metabolic Syndrome due to PTH and the Effects of Adjusting for Individual Components of the Metabolic Syndrome on PTH Odds Ratios in a Logistic Regression Analysis

Model number	Independent variables	Odds ratios	95% CIs	p-values
1	Log PTH	2.48	1.01, 6.08	0.04
2	Log PTH Triglyceride ≥ 1.7 mmol/L	2.91 11.9	1.08, 7.83 7.19, 19.6	0.03 <0.0001
3	Log PTH HDL-C < 1.3 mmol/l (females) or < 1.0 mmol/L (males)	3.08 13.3	1.09, 8.76 8.41, 21.0	0.03 <0.0001
4	Log PTH Glucose ≥ 5.6 mmol/L	2.28 9.58	0.87, 5.97 5.90, 15.6	0.09 <0.0001
5	Log PTH Diastolic bp ≥ 85 mm/Hg	2.76 5.85	1.05, 7.24 3.92, 8.73	0.04 <0.0001
6	Log PTH Systolic bp ≥ 130 mm/Hg	1.76 8.32	0.66, 4.65 5.28, 13.1	0.25 <0.0001
7	Log PTH Waist ≥ 94 or ≥ 90 cm for BA or Indian males or ≥ 80 cm for females	2.03 9.69	0.82, 5.06 4.55, 20.6	0.13 <0.0001
8	Log PTH Systolic bp ≥ 130 mm/Hg Waist ≥ 94 or ≥ 90 cm for BA or Indian males or ≥ 80 cm for females	1.43 8.46 10.3	0.53, 3.85 5.32, 13.5 4.66, 22.7	0.48 <0.0001 <0.0001

All models adjusted for age, gender, ethnicity, BMI and 25(OH)D with metabolic syndrome as dependent variable.

PTH pmol/L, HDL-C mmol/L, Glucose mmol/L, Blood pressure mmHg, Waist cm

Adding both these variables to the regression model at the same time attenuates the p-value for the PTH OR to a greater extent than adding each variable on its own (see Table 4.7, model 8). This suggests that the effects of each variable are additive and independent. Addition of the other metabolic syndrome components to the logistic regression model does not attenuate the p-value/OR for PTH and in some cases increases it. The exception is glucose, which does have a weak effect on the p-value for PTH, increasing it from 0.04 to 0.09 (see Table 4.7, model 4).

4.4 DISCUSSION

There are no previous studies from sub-Saharan Africa that have examined the relationship between 25(OH)D, PTH and Met S. In this study we investigated whether 25(OH)D or PTH is associated with Met S or its components. Our results show a significant association of PTH, but not 25(OH)D, with Met S. Higher PTH levels were associated with increased systolic and diastolic blood pressure and reduced insulin resistance as assessed by the HOMA index and an increased

odds ratio for Met S. Furthermore our results suggest that neither 25(OH)D nor PTH contribute to ethnic differences in the prevalence of Met S between Black African and Indian subjects.
Our observations of a lack of association of 25(OH)D with Met S are in line with those of Scragg et al who observed no association between 25(OH)D status and type 2 diabetes in non-Hispanic black subjects, but did observe a negative associations between 25(OH)D and risk of diabetes in Mexican Americans and non-Hispanic white populations (247). Similarly, in a single study from India, Majumdar et al showed that although 25(OH)D insufficiency was highly prevalent, it was not associated with Met S or insulin resistance (253).

The results of studies on the association of 25(OH)D and PTH with Met S, components of Met S or related disorders have been inconsistent. In a population based cross sectional study of US men and women over 20 years-of-age (NHANES III, 1988-94) the prevalence of Met S and its components fell across increasing quartiles of 25(OH)D after adjustment for age, race, sex, income, lifestyle factors, calcium and energy intake (387). This study also showed that the odds ratio for Met S increased with increasing PTH in older men only. In a study carried out in an aging European population, investigators showed that the risk for Met S decreased across increasing quintiles of 25(OH)D but could show no association of PTH with Met S (288). Neither of these studies adjusted for BMI. However, Reis et al. showed a lack of association between serum 25(OH)D and Met S in an adult Caucasian population (255). Our population, particularly the Black African subset, was not particularly vitamin D deficient overall (median 25(OH)D in Black Africans was 71 nmol/L) and 25(OH)D may be more strongly associated with Met S in a deficient population. However, this cannot explain the lack of association of 25(OH)D with Met S in the Indian group, as serum 25(OH)D levels were significantly lower in this population compared to the Black African cohort.

A number of other studies have implicated PTH as being associated with Met S rather than 25(OH)D (288, 382). Several lines of evidence support a role of PTH in increasing the risk of CVD. It has been associated with increased cardiovascular mortality in selected population groups (254, 388, 389) and with increased coronary heart disease in a younger group (390). This increased risk may be mediated via its effects on blood pressure, insulin resistance, hyperglycaemia and low HDL-C (255, 387). In multivariate regression models we confirmed a positive

relationship between PTH and blood pressure but could not verify any relationship between PTH and blood glucose or lipid levels, although significant associations were observed in univariate analyses. The negative relationship that we observed between PTH and the HOMA index was only observed after adjusting for confounding variables within a multivariate regression analysis. In a univariate analysis PTH correlated positively with HOMA. Derangements in glucose metabolism in primary hyperparathyroidism are well described with increased PTH increasing glucose intolerance (261, 387, 391, 392). A few studies have described a positive relationship between PTH and insulin resistance similar to our observation in univariate analyses. Some investigators have postulated that this relationship may be secondary to hypercalcaemia as it is absent in normocalcaemic subjects (393, 394). Frost et al showed a significant negative association of PTH with insulin resistance in young men (395). The negative relationship we have noted between PTH and HOMA in a multivariate analysis may be for a number of reasons: ethnic differences, the fact that our subjects were all normocalcaemic or the adjustment for confounders. In addition to its possible effect on insulin sensitivity, clinical evidence also supports a role of PTH in increasing blood pressure (383) and observational studies have linked elevated PTH levels to an increased risk of hypertension, left ventricular hypertrophy, and cardiovascular morbidity and mortality (389). It is thought that PTH mediates its effect by directly increasing the secretion of aldosterone from the adrenal glands and indirectly by activating the renin-angiotensin system (396).

Logistic regression models demonstrated that the increased risk of Met S in subjects with higher PTH levels is largely due to the positive and independent relationships of systolic blood pressure and waist circumference with PTH. Previous studies have also shown a positive association of PTH with obesity (389, 397). Furthermore, body weight changes correlate positively with changes in serum PTH levels (398, 399) suggesting that obesity may be causative for hyperparathyroidism. There are no studies in the literature showing that PTH is secreted by adipocytes, but there are studies that demonstrate that PTH does modify adipocyte function. These effects include the inhibition of adipocyte lipoprotein lipase activity (280) and the attenuation of insulin signalling (400). However, both these actions of PTH would limit rather than augment triglyceride deposition within adipose tissue. It is possible that increased adiposity may lead to greater production of PTH by the parathyroid, and it is interesting to note that

studies have shown a positive correlation between serum leptin and PTH concentrations (397).

Our data show that Indian ethnicity is associated with increased risk for Met S and most of its components. As a group, Indians tend to be insulin resistant and at high risk for diabetes and premature coronary heart disease when compared to other ethnic groups (401). This was true of this study cohort. This may be partly explained by high visceral fat content (402). In the present study ethnic differences in the levels of Met S related metabolic variables and the prevalence of Met S were investigated, using multiple regression and logistic regression analyses and were found not to be related to ethnic differences in PTH or 25(OH)D levels.

This study is limited by the cross-sectional nature of the data so we cannot draw any conclusions about causality. Another limitation is that blood samples were collected over several months, and vitamin D is known to display seasonal variation. Furthermore, study subjects were not selected randomly and this may have introduced some level of selection bias. Finally, 25(OH)D levels were not measured using the reference method of liquid chromatography-tandem mass spectrometry (LC/MS/MS), however the technique used in this study (HPLC) has been shown to correlate well with the LC/MS/MS method (125).

4.5 CONCLUSIONS

In conclusion, PTH but not 25(OH)D is associated with Met S in our Black African and Indian populations. The PTH effect is largely via its impact on blood pressure and its relationship with waist circumference. The relationship between PTH and insulin resistance needs to be investigated further, as does the mechanism responsible for causing serum PTH levels to rise with increased adiposity.

We have shown that although there are clear differences in 25(OH)D concentrations between both population groups this does not contribute to ethnic differences in the Met S. We also showed in the first study that Indian subjects have greater total body fat compared to Black African subjects. The aim of the third study was therefore to investigate body composition differences in both ethnic groups in more detail and to identify how body composition and 25(OH)D affect BMD in both these population groups.

CHAPTER 5

5. THE ASSOCIATION BETWEEN BODY COMPOSITION, 25(OH)D AND PTH, AND BONE MINERAL DENSITY IN BLACK AFRICAN AND INDIAN POPULATION GROUPS

ABSTRACT

As there are little data on the contribution of body composition to bone mineral density (BMD) in non-Caucasian populations we investigated the contribution of body composition, and possible confounding of 25(OH)D and PTH, to BMD at various skeletal sites in both groups of subjects.

BMD, body fat and lean mass were measured using dual x-ray absorptiometry (DXA) and abdominal fat distribution by ultrasound in 714 healthy subjects, ages 18-65 years.

Whole body (subtotal), hip (H), femoral neck (femoral) and lumbar spine (lumbar) BMD were significantly higher in Black African than Indian subjects ($p<0.001$ for all). Whole body lean mass positively associated with BMD at all sites in both ethnic groups ($p<0.001$ for all), and partially explained the higher BMD in Black African females compared to Indian females. Whole body fat mass correlated positively with lumbar BMD in Black African ($p=0.001$) and inversely with subtotal BMD in Indian subjects ($p<0.0001$). Visceral adiposity correlated inversely with subtotal BMD in the Black African ($p=0.037$) and with lumbar BMD in the Indian group ($p=0.005$). No association was found between serum 25(OH)D and BMD. PTH was inversely associated with hip BMD in the Black African group ($p=0.01$) and with sub total ($p=0.002$), hip ($p=0.001$) and femoral BMD ($p<0.0001$) in the Indian group.

Significant differences in whole body and site-specific BMD between Black African and Indian groups were observed with lean mass the major contributor to BMD at all sites in both groups. The contribution of other components of body composition differed by site and ethnic group.

5.1 INTRODUCTION

South Africa, a low to middle income country undergoing an epidemiological transition, is characterized by a high rate of infectious diseases, malnutrition and an increasing prevalence of non-communicable diseases (6). There are limited comparative bone mineral density (BMD) data on non-Caucasian population groups living in countries that are part of the epidemiological transition, although it is known that osteoporotic fractures are an important cause of disability and mortality world-wide (403). Data from the late 1960's have reported that fracture rates in white, Indian and mixed ancestry South African populations are similar to that in Western countries, while fractures of the hip are reportedly less common in Black African subjects (404). A recent comparison of fractures in adolescent mother-daughter pairs noted that white adolescents and their mothers had more fractures compared to black adolescent mother-daughter pairs (405). Ethnic differences in BMD have been reported with black South African females having higher BMD at the femoral neck, than their white counterparts, and similar BMD at the lumbar spine and distal radius,(220, 406) while a more recent study has reported higher BMD at the femoral and hip, but lower lumbar BMD in Black African than white women (407). It is possible that ethnic differences in BMD contribute to differences in fracture rates however to our knowledge similar data are not available for Black African men or Indian subjects.

Differences in body composition may contribute to differences in BMD, as positive associations between body weight and body mass index, and BMD, as well as an increased fracture risk with low BMI, have been demonstrated (408). Significant sex and ethnic differences exist in the prevalence of obesity (9) and in adipose tissue distribution, with Black African women having lower visceral adiposity than Indian women (402) and white South African women (409). Increased weight is associated with increased BMD due to the additional mechanical loading on the bone which occurs with increased weight bearing, as well as via muscle-mediated effects of physical exercise (212). However the relationship between fat mass and BMD is less clear with some studies reporting a positive association (410) and other studies demonstrating a negative effect on BMD (411). Furthermore, visceral and subcutaneous adipose tissue which are distinct in terms of their secretion of steroid hormones and adipokines (27) as well as inflammatory cytokines (412) may also affect BMD differently (37).

Inverse associations between BMD and both visceral adiposity and sub-cutaneous adiposity have been demonstrated in several groups including African-Americans and Caucasians (413) obese premenopausal women (414) and obese adolescent girls (412). In contrast, others have shown a positive association between sub-cutaneous adiposity and BMD in young females (412). A recent South African study reported that after adjusting for a number of variables including visceral adiposity and sub-cutaneous adiposity, neither visceral adiposity or sub-cutaneous adiposity were associated with BMD in white or African women (407).

Obesity is associated with hypovitaminosis D (316, 350) and secondary hyperparathyroidism (316), both of which may affect BMD negatively. We have previously reported a 28.6% prevalence of vitamin D deficiency in an Indian population resident in Johannesburg, compared to 5.1% in African subjects (415). In the same study we showed an inverse association between BMI and 25(OH)D and a positive relationship between PTH and waist circumference. Whether 25(OH)D or PTH levels influence BMD in these population groups has not been investigated.

The aims of this study were to:

(i) investigate ethnic and sex differences in BMD, fat mass and lean mass between Black African and Indian men and women
(ii) identify how body composition contributes to BMD at various skeletal sites in both ethnic groups; and
(iii) determine whether the effects of body composition on BMD at various skeletal sites are mediated via PTH and/or 25(OH)D

5.2 METHODS

Please refer to chapter two for a description of the following methods: weight, height, and waist circumference, visceral and subcutaneous adiposity, whole body fat, bone area and bone mineral density of whole body, lumbar spine, femoral neck and hip, serum calcium, phosphate and creatinine as well as plasma PTH and 25(OH)D determination of smoking status and alcohol use.

Seven hundred and fourteen subjects for whom we had complete data were included in this study, 371 Black African and 343 Indian, of whom 340 were males and 374 females.

5.2.1 Statistical Analysis

Data are presented as mean ± SD for normally distributed data and median (interquartile range (IQR)) for data that was not normally distributed. The latter variables were log transformed before analysis except for subtotal fat which was transformed by square root transformation. Exclusion of subjects with TSH above the laboratory reference range made no difference to the analyses and therefore these were included in all analyses. Only one participant reported the use of corticosteroids. Analysis of variance (ANOVA) with Bonferroni correction was used to compare BMD and body composition by ethnicity and gender. All bone data were compared after adjustment for height, weight, age and bone area. Pearson's correlations were used to examine the strength of the association of BMD with variables that have been shown to affect BMD (412, 416-421). Multivariable regression analyses were performed to determine the variables that made a significant contribution to sub-total, lumbar, femoral and hip BMD in the two ethnic groups. In the regression models, BMDs were used as dependent variables after log transformation, except for the lumbar BMD. Backwards stepwise regression analysis was performed until only variables with a $p<0.05$ were left in the model. Smoking status was added to regression models for all subjects as it may have been a possible confounder. Alcohol consumption was used in the Black African model only as only 4.4% of the Indians reported using alcohol.

To test if the effects of body composition were mediated by PTH, we noted whether removal of PTH from the final regression model uncovered significant associations of body composition variables with BMD. Since we did not collect data on menopausal status, age was used as a categorical variable with those less than 50 coded as 1 and those 50 years or more coded as 2. There was no collinearity between the various body composition variables, as evidenced by variance inflation factors of <10.0. We tested for interaction of both gender and ethnicity with body composition and BMD and noted that there was no interaction

Analysis of covariance (ANCOVA) was used to determine if any variables modulated the ethnic differences observed in BMD within each gender group. Thus, ANCOVA models were set up using ethnicity as the grouping variable and BMD as the dependent variable. Covariates were selected based on their ability to influence BMD and their observed differences between the 2 ethnic groups. These covariates were lean mass in females, sub-cutaneous adiposity and fat mass in males, and 25(OH)D in both groups. Each covariate was added individually to each model and the effects on the F- and p-values for ethnicity were noted. If more

than one covariate was observed to attenuate the F- and p-values in one model they were added together in a further ANCOVA.

5.3 RESULTS

5.3.1 Ethnic Differences in Body Composition and BMD

Descriptive and body composition characteristics by gender and ethnicity are presented in Table 5.1. The mean age of all the subjects was 42.6±13 years, and there was no difference in age or height between the ethnic groups. Black African females were significantly heavier and had a greater BMI than Indian females, whilst Black African males weighed less, had a lower BMI and a lower waist circumference than Indian males. Waist-to-hip ratio was significantly higher in the Indian compared to the Black African groups, irrespective of sex. In males, fat mass was lower in Black African than in Indian subjects but lean mass was not different. Lean mass was significantly higher in Black African than Indian females, whilst fat mass was not different.

Visceral adiposity was significantly higher in Black African females and Indian males compared to their same sex counterparts, and sub-cutaneous adiposity was significantly higher in Indian males compared to Black African males. Visceral adiposity/sub-cutaneous adiposity ratio was higher in Black African males and females compared to Indian groups.

Subtotal and lumbar bone areas were higher in Black African females and males compared to their same sex Indian counterparts. All BMD measures were significantly higher in Black African males and females compared to their Indian counterparts. These differences remained significant after adjusting for age, height, weight and site-specific bone area.

As body weight and BMI were significantly higher in Indian males compared to Black African males, we assessed BMD for 100 men of similar BMI: including 44 Black African men with a mean BMI of 26.1 (25.4, 26.6) and 56 Indian men with a mean BMI of 25.6 (25.4, 26.2), p=0.6. The BMD was significantly higher at all sites in Black African men ($P<0.0001$ for subtotal, hip and femoral BMD and $P=0.0002$ for lumbar BMD).

5.3.2 Gender Differences in Body Composition and BMD

Males were taller than females within their respective ethnic groups. Black African females were heavier, had a greater BMI and larger waist circumference than Black African males, whilst Indian females were lighter and had a smaller waist circumference than Indian males. Waist-hip ratio and lean mass were higher, and fat mass lower, in males compared to females in both ethnic groups. Although visceral adiposity was not significantly different between Black African males and females, it was higher in Indian males than females. In both ethnic groups, sub-cutaneous adiposity was higher and visceral adiposity/sub-cutaneous adiposity ratio was lower in females than males.

After adjusting for age, height, weight and site-specific bone area, subtotal BMD was significantly higher in males compared to females of both ethnic groups.

Table 5.1: Descriptive Characteristics of African and Indian Male and Female Participants

	BA Females (n=187)	Indian Females (n=187)	P-value	BA Males (n=181)	Indian Males (n=160)	P-value
Age (yrs.)	41.7±13.1	43.8 ± 12.7	0.08	41.7±13.2	43.0±13.2	0.36
Height (cm)	158 ±6.90	158±5.98	0.6	171±7.32***	171±6.18###	0.99
Weight (kg)	75.5 (60.8,90.1)	68.4 (56.6,80.5)	0.001	67.7 (59.7,79.9)*	77.3 (66.8,88.4)###	<0.0001
BMI (kg/m^2)	29.9 (24.3, 35.3)	27.1 (23.0, 32.8)	0.0007	23.3 (20.2,27.3)***	26.2 (23.7,30.4)	0.002
Waist (cm)	94.4±15.7	92.5±16.9	0.9	87.1±13.9***	98.5±13.9##	<0.0001
WHR	0.85±0.09	0.87±0.12	0.04	0.89±0.08***	0.96±0.08###	<0.0001
STLM (kg)	38.4 (34.1,42.7)	34.4 (30.3,39.3)	<0.0001	46.9 (42.9,52.1)***	48.8 (42.9,54.4)###	0.89
STFM(kg)	28.3 (20.9,30.0)	28.4 (20.1,35.1)	0.38	11.9 (7.27,21.1)***	20.1 (15.4,21.3)###	<0.0001
VAT (cm)	5.13±1.76	4.19±1.73	<0.0001	4.98±1.91	5.84±1.96###	0.0001
SCAT (cm)	3.20 (2.26,4.23)	3.34 (2.49,4.07)	0.61	1.67 (1.29,2.33)***	2.81 (2.02,3.56)#	<0.0001
STBMD (g/cm^2)	0.966 (0.953,0.978)	0.912 (0.895,0.925)	<0.0001	1.052 (1.039,1.084)***	1.005 (0.997,1.028)###	<0.0001
ST bone area (cm^2)	1713± 153	1647±159	<0.0001	1923± 183*	1905±177##	<0.0001
LSBMD (g/cm^2)	0.702 (0.691, 0.713)	0.648 (0.636, 0.659)	<0.0001	0.695 (0.683, 0.707)	0.671 (0.659, 0.683)	0.02
LS bone area (cm^2)	48.8± 5.58	46.9±5.31	0.007	58.6±7.13***	54.9±5.90###	<0.0001
HBMD (g/cm^2)	0.998 (0.909,1.088)	0.887 (0.803,0.978)	<0.0001	1.068 (0.934,1.162)	0.964 (0.882,1.051)	<0.0001
H bone area (cm^2)	32.9± 3.39	33.4± 4.09	0.9	43.1±4.50***	43.2±4.58###	0.9
FNBMD (g/cm^2)	0.919 (0.830,0.995)	0.777 (0.692,0.859)	<0.0001	0.938 (0.826,1.026)	0.793 (0.723,0.873)	<0.0001
FN bone area (cm^2)	4.92±0.359	4.92±0.374	0.9	5.51±0.418***	5.56±0.398###	0.9
PTH (pmol/L)	5.30 (3.80, 5.90)	5.00 (3.70, 7.20)	0.8	4.30 (2.00,5.60)**	4.60 (3.60,6.43)	0.16
25(OH)D (nmol/L)	58.3 (42.9, 85.6)	35.7 (23.0, 54.5)	<0.0001	72.7 (51.1,94.1)*	45.4 (33.6,62.7)##	<0.0001
eGFR (ml/min/1.73m^2)	87.4(77.4,103)	92.8(81.6,108)	<0.0001	88.7(74.5,103)	84.2(72.7,96.3) ###	0.004

Table 5.1 continued

	BA Females (n=187)	Indian Females (n=187)	P-value	BA Males (n=181)	Indian Males (n=160)	P-value
Ccalcium (mmol/L)	2.27±0.10	2.26±0.11	0.96	2.28±0.08	2.26±0.10	0.06
Phosphate (mmol/L)	1.09±0.17	1.11±0.15	0.11	1.05±0.18	1.05±0.15	0.79
TSH (miU/L)	1.96±1.16	2.41±1.62	0.002	1.97±1.09	2.44±1.71	0.002

Key: Data presented as mean±SD if normally distributed and median (IQR) if non-normally distributed. BA male vs. BA female, *p<0.05, **p<0.001, ***p<0.0001; AI male vs. AI female, $^{\#}$p<=0.05, $^{\#\#}$p<0.001 $^{\#\#\#}$p <0.0001; all bone area adjusted for height, age and weight. All bone mineral density data adjusted for bone area as well. VAT=Visceral adipose tissue thickness, SCAT=subcutaneous adipose tissue thickness, STFM=subtotal fat mass, STLM=subtotal lean mass, STBMD=subtotal BMD, LSBMD=lumbar BMD, HBMD=hip BMD, FNBMD=femoral neck BMD, PTH=parathyroid hormone, ST=sub-total, LS=Lumbar spine, H=Hip, FN=Femoral neck.BA=Black African

5.3.3 **Biochemistry**

Mean plasma calcium and phosphate concentrations were within normal reference ranges for our laboratory and were not significantly different between ethnic or gender groups. Median eGFR was within laboratory reference ranges for both genders, but significantly lower in Black African females compared with Indian females, and higher in Black African males compared with Indian males. It was not significantly different between Black African males and females (p=0.87) but was higher in Indian females compared to Indian males (p<0.0001) PTH levels were not significantly different between ethnic groups but were significantly lower in Black African males than females. 25(OH)D levels were significantly higher in Black African males and females compared to their Indian counterparts, and were higher in males than females in both ethnic groups. TSH was not significantly different between males and females for each ethnic group but was significantly higher in Indian females compared to Black African females (p=0.002) and in Indian males compared to Black African males.

5.3.4 **Smoking Status and Alcohol Use**

There were more smokers amongst the Black African than the Indian subjects (33.7% vs. 16.2%, p<0.0001) and among males than females (Black African: 53.3% vs. 14.9%, p<0.0001 and Indian: 29.5% vs. 5.4%, p<0.0001). More Black African than Indian subjects consumed alcohol (37.3% vs. 4.4%, p<0.0001) and

males in each group consumed more alcohol than did their female counterparts (Black African: 45.3% vs. 29.6%, p=0.002 and Indian: 8.3% vs. 1.07%, p=0.001).

5.3.5 Univariate Correlation Analysis

Pearson correlations between BMD and all other variables are presented in Table 5.2. Scatter plots are presented for subtotal BMD vs. all STLM, VAT and SCATvariables. Lean mass was significantly correlated with all bone parameters in the two ethnic groups (all p<0.0001). Fat mass was positively associated with hip, femoral neck and lumbar BMD in both ethnic groups. Visceral adiposity correlated with hip BMD in the Black African group and with all BMD sites except lumbar spine in the Indian group was significantly correlated with sub-total, femoral neck and lumbar BMD in the Black African group and with BMD at all sites in the Indian group. 25(OH)D was significantly correlated with sub-total and hip BMD in both groups. PTH was inversely correlated with sub-total BMD in the Black African group and was not significantly correlated with any BMD measures in the Indian group.

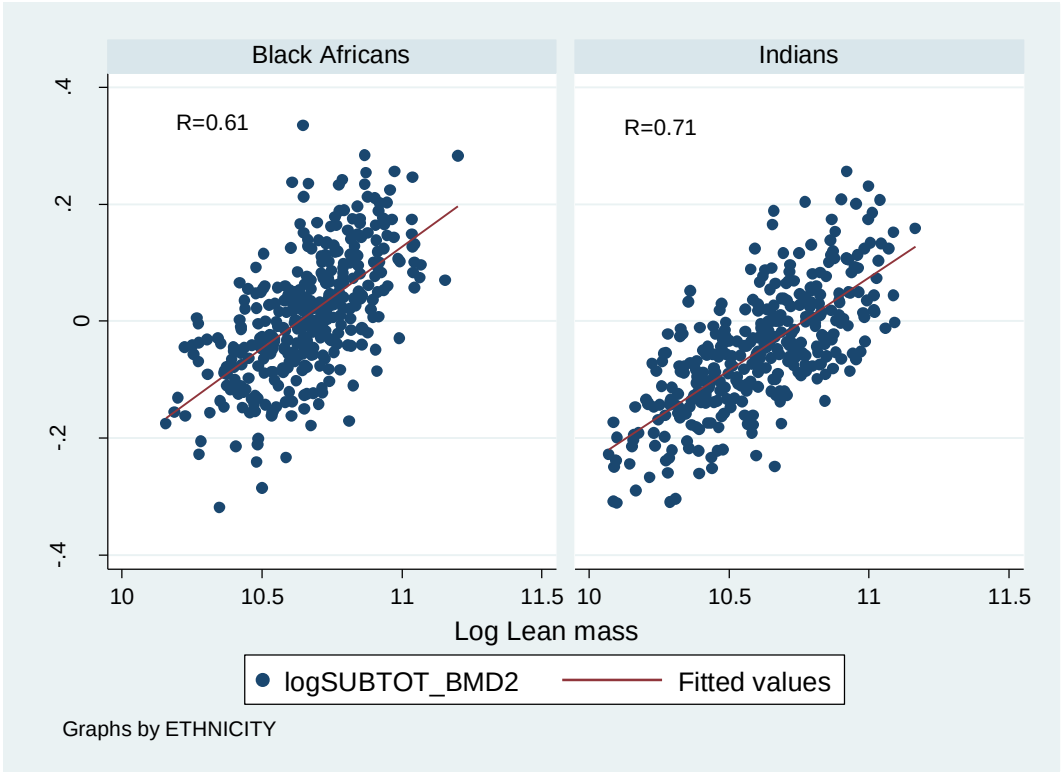


Figure 5.1 Scatter plot for Log Subtotal BMD (g/cm2) vs. Log Lean mass(kg).

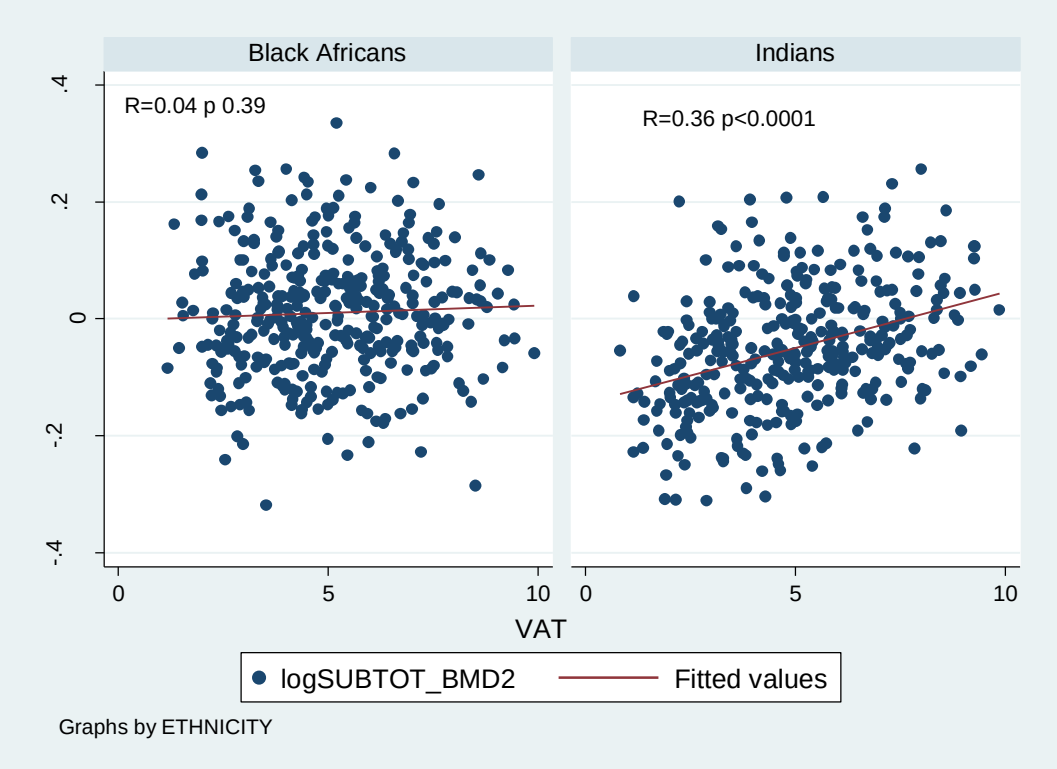


Figure 5.2 Scatter plot for Log Subtotal BMD(g/cm2) vs. Visceral adipose tissue(VAT)(cm).

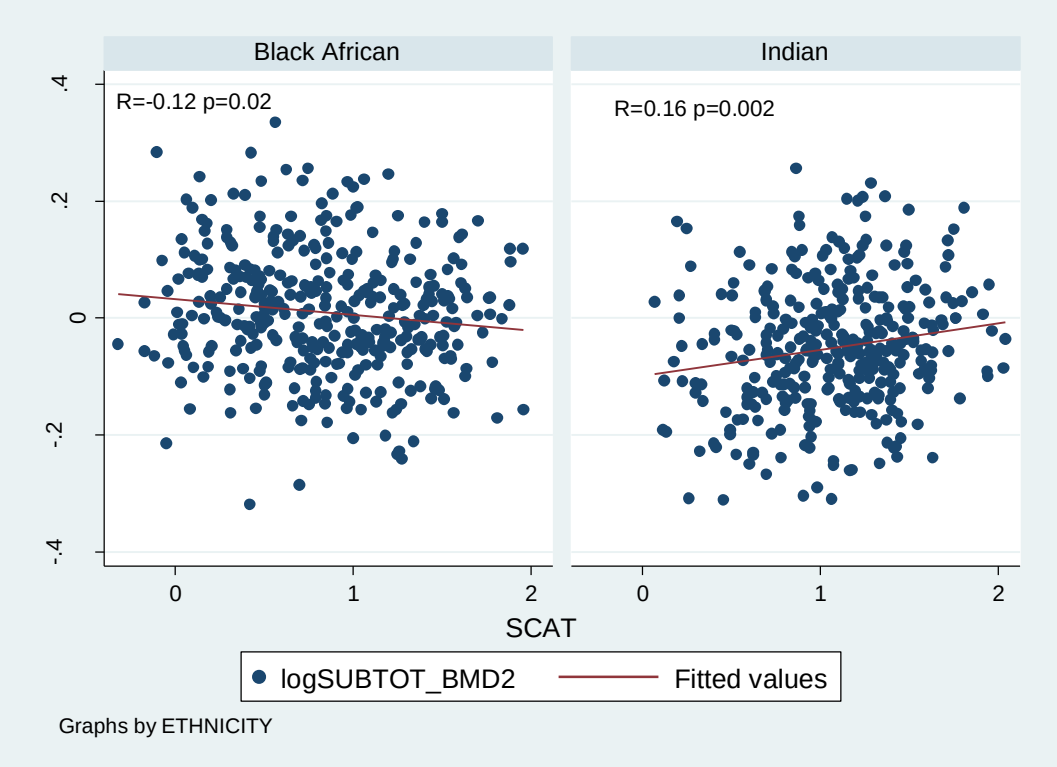


Figure 5.3 Scatter plot for Log Subtotal BMD(g/cm2) vs. Log Subcutaneous adipose tissue(SCAT)(cm).

Table 5.2: Pearson Correlations for BMD by Ethnicity

Variables	BA (n=371)				Indians (n=343)			
	STBMD	**HBMD**	**FNBMD**	**LSBMD**	**STBMD**	**HBMD**	**FNBMD**	**LSBMD**
Age (years)	-0.004 (0.94)	-0.03 (0.59)	-0.22 (0.0001)	-0.08 (0.13)	-0.04 (0.51)	-0.02 (0.71)	-0.17 (0.001)	-0.06 (0.24)
Height (cm)	0.47 (<0.0001)	0.20 (<0.0001)	0.09 (0.06)	0.15 (0.005)	0.54 (<0.0001)	0.27 (<0.0001)	0.19 (0.04)	0.12 (0.02)
25(OH)D (nmol/L)	0.09 (0.06)	0.05 (0.32)	0.02 (0.70)	0.02 (0.65)	0.15 (0.004)	0.11 (0.04)	0.08 (0.19)	-0.02 (0.63)
Calcium (mmol/L)	0.04 (0.50)	0.006 (0.90)	-0.01 (0.81)	-0.02 (0.68)	-0.11 (0.007)	-0.06 (0.23)	-0.06 (0.22)	0.03 (0.53)
Phosphate (mmol/L)	-0.13 (0.01)	-0.13 (0.01)	-0.02 (0.75)	-0.08 (0.12)	-0.07 (0.22)	-0.07 (0.72)	-0.06 (0.25)	-0.03 (0.55)
PTH (pmol/L)	-0.12 (0.02)	-0.08 (0.12)	-0.06 (0.18)	-0.01 (0.82)	-0.06 (0.24)	-0.01 (0.77)	-0.08 (0.11)	0.05 (0.38)
eGFR (ml/min/1.73m$_2$)	-0.17 (0.0080)	-0.15 (0.002)	-0.04 (0.46)	-0.09 (0.08)	-0.21 (0.0001)	-0.13 (0.01)	0.02 (0.71)	0.002 (0.97)
STFM (kg)	-0.08 (0.11)	0.16 (0.002)	0.15 (0.005)	0.15 (0.003)	0.09 (0.08)	0.29 (<0.0001)	0.26 (<0.0001)	0.16 (0.002)
STLM (kg)	0.61 (<0.0001)	0.54 (<0.0001)	0.36 (<0.0001)	0.28 (<0.0001)	0.71 (<0.0001)	0.55 (<0.0001)	0.39 (<0.0001)	0.25 (<0.0001)
VAT (cm)	0.04 (0.39)	0.16 (0.002)	0.07 (0.16)	0.06 (0.22)	0.36 (<0.0001)	0.35 (<0.0001)	0.20 (0.0001)	0.06 (0.21)
SCAT (cm)	-0.12 (0.02)	0.08 (0.14)	0.15 (0.005)	0.09 (0.07)	0.16 (0.002)	0.29 (0.002)	0.28 (<0.0001)	0.14 (0.006)

Key: BA=Black African Data given as R-values (p-values); STLM=subtotal lean mass, STFM=subtotal fat mass, VAT=visceral adiposity, SCAT=subcutaneous adipose tissue. STFM was transformed to normality, by taking the square root and the following were log transformed 25(OH)D, PTH, STLM, SCAT and all BMD except for L BMD.

5.3.6 Multivariable Regression Models for BMD

In the Black African group, 43% of the variance in sub-total BMD was explained by lean mass and visceral adiposity compared to the Indian group where 54% of the variance was explained by lean mass and fat mass. At the hip, lean mass and PTH explained 32% of the variance in BMD in the Black African group, whilst 39% of the variance was explained by the same variables in the Indian group. Age, lean mass and fat mass explained 18% of the variance in femoral BMD in the Black African group, while 29% of the variance in femoral BMD in the Indian group was explained by age, lean mass and PTH. At the lumbar spine 12% of the variance in BMD was explained by age, lean mass and fat mass in the Black African group, whilst 12% of the variance in BMD was explained by age, lean mass and visceral adiposity in the Indian group. We tested for interaction of body composition variables with both gender and ethnicity and noted a lack of significant interaction in both sets of analyses. The effect of body composition was not mediated by PTH,

as removal of PTH from regression models did not alter the β coefficient or p values for any body composition variables.

Table 5.3: Multivariable Regression for Africans and Indians

Ethnic group	Dependent variables	Independent variables	β coefficient (95% CIs)	P-value for β	Unadjusted R^2 (P value)
Black African n=371	**STBMD**	Gender STLM (VAT	0.04 (0.02, 0.06) 0.29 (0.24, 0.35) -0.005 (-0.01,-0.0003)	0.001 <0.0001 0.037	0.41 (<0.0001)
	HBMD	STFM STLM PTH	0.02 (0.0003, 0.04) 0.49 (0.41, 0.57) -0.11 (-0.12, -0.02)	0.028 <0.0001 0.01	0.33 (<0.0001)
	FNBMD	Age STFM STLM	-0.05 (-0.08, -0.02) 0.05 (0.03, 0.07) 0.38 (0.30, 0.48)	<0.0001 <0.0001 <0.0001	0.18 (<0.0001)
	LSBMD	Age STFM STLM	-0.05 (-0.08, -0.02) 0.03 (0.01, 0.05) 0.20 (0.13, 0.28)	<0.0001 0.001 <0.0001	0.12 (<0.0001)
Indian n=343	**STBMD**	Gender STLM STFM PTH	-0.05 (-0.08,- 0.02) 0.42 (0.34, -0.48) 0.03(0.002,0.06) -0.06 (-0.09,-0.02)	0.002 <0.0001 0.032 0.002	0.54 (<0.0001)
	HBMD	Gender STLM PTH	-0.11(-0.16,-0.07) 0.61 (0.52, 0.71) -0.12 (-0.19, -0.05)	0.038 <0.0001 0.001	0.39 (<0.0001)
	FNBMD	STLM PTH	0.58 (0.48, 0.68) -0.16 (-0.25,-0.08)	<0.0001 <0.0001	0.29 (<0.0001)
	LSBMD	STLM VAT	0.36 (0.26, 0.46) -0.013 (-0.02, -0.003)	<0.0001 0.005	0.12 (<0.0001)

Key: STBMD=sub-total BMD; HBMD=hip BMD; FNBMD=femoral neck BMD; LSMD=lumbar spine BMD; STFM=sub-total fat mass; STLM=subtotal lean mass; VAT=visceral adipose tissue thickness; SCAT=subcutaneous adipose tissue thickness; PTH=parathyroid hormone. STFM was transformed to achieve normality by taking the square root, the following were log transformed: subtotal, hip, femoral and hip BMD, PTH, 25(OH)D and STLM. Units:BMD g/$cm^{2,}$ PTH pmol/L, Age years VAT cm, STLM kg, STFM kg,

5.3.7 ANCOVA Models for Detecting Variables that may contribute to Ethnic Differences in BMD

Results are presented in Table 5.4 for sub-total and hip BMD only since the trends observed for femoral and lumbar BMD were very similar to those for hip and sub-total BMD within each gender. In females, the effect of ethnicity on BMD was attenuated by the addition of lean mass into the model at all sites, particularly at the lumbar BMD where ethnicity was no longer significant when including lean

mass in the model; addition of lean mass reduced F-value for ethnicity from 10.4 (p=0.001) to 1.80 (p=0.18). It appears that the effect of visceral adiposity on the ethnic difference in BMD seen in females is largely due to its correlation with lean mass (r=0.56, p<0.0001). Thus, addition of visceral adiposity to all ANCOVA models already containing lean mass as a covariate did not substantially attenuate the F-value for ethnicity, whilst the F-value for visceral adiposity was lowered and became insignificant for all skeletal sites. In males the ethnicity effect was attenuated slightly by the addition of 25(OH)D to the ANCOVA model for each skeletal site.

Table 5.4: Contribution of Body Composition and 25(OH)D to Ethnic Differences in BMD at Selected Skeletal Sites in Males and Females as Assessed using ANCOVA

Skeletal site; Gender	Model Number	Grouping Variable* and Covariates	F-value; P-value
Sub-total BMD; Female	1	Ethnicity	42.7; <0.0001
	2	Ethnicity STLM	11.2; 0.0009 115; <0.0001
	3	Ethnicity VAT	26.4; 0.0001 27.1; <0.0001
	4	Ethnicity STLM visceral adiposity	10.8; 0.001 141; <0.0001 1.41; 0.23
Sub-total BMD; Male	1	Ethnicity	26.4; <0.0001
	2	Ethnicity 25(OH)D	12.9; 0.0004 0.65; 0.006
Hip BMD; Female	1	Ethnicity	42.1; <0.0001
	2	Ethnicity STLM	11.8; 0.0006 151; <0.0001
	3	Ethnicity VAT	23.3; <0.0001 48.2; 0.02
	4	Ethnicity STLM VAT	10.2; 0.002 95.1; <0.0001 1.83; 0.18
Hip BMD; Male	1	Ethnicity	41.4; <0.0001
	2	Ethnicity 25(OH)D	23.5; <0.0001 6.68 <0.01

*Within each model the grouping variable is ethnicity; for each skeletal site the first model is an ANOVA and subsequent models are ANCOVAs; STLM=subtotal lean mass, STFM=subtotal fat mass, VAT=visceral adiposity. STFM was transformed to achieve normality by taking the square root, and the following were log transformed 25(OH)D, STLM, SCAT and all BMD. BMD g/cm2, VAT cm, SCAT cm, 25(OH)D nmol/L

5.4 DISCUSSION

In this group of apparently healthy adults we have shown that BMD is significantly higher at all sites in Black African compared to Indian subjects, and that lean mass was a significant contributor to all BMD sites in both ethnic groups. Differences in lean mass between Black African and Indian females mediated the difference in lumbar BMD between the ethnic groups in female subjects and contributed to differences in sub-total, hip and femoral BMD, whilst in males differences 25(OH)D contributed to a small degree to ethnic differences in BMD. Compared to the other BMD sites we were able to explain very little of the variance in lumbar BMD using the variables collected in this study.

Our findings of a positive association between lean mass and BMD are in keeping with those of Amarendra et al (416) in their study of Indian men, Chantler et al in African women (407) and others (422). This is the first report of the significant contribution of lean mass to BMD in black South African men. The positive association between lean mass and BMD remained after adjustment for fat mass, suggesting that the effects of lean mass on BMD are not entirely due to mechanical loading. Other contributory factors may include the pull of muscle contraction on bone and cytokines from muscle that influence muscle-bone cross talk (423).

Several investigators have shown that black men and women have greater bone mass whilst Indians have lower bone mass than white subjects (407, 424, 425). The greater bone density in Black Africans may be protective of fractures given that fracture rates are similar in Indian and Caucasian women (426) whilst Black African have lower fracture rates compared to other ethnic groups (404). Our study showed that the Black African group had greater BMD at all four sites compared to the Indian group. In a comparison of BMD across four ethnic groups, Nam et al attributed the greater BMD in African American women compared to Asian women to differences in weight (427). In our study, body weight was greater in the Black African compared to Indian females, largely due to greater lean mass. When adjusting for differences in weight between the ethnic groups, BMD at all sites was still significantly higher in the Black African females, however the inclusion of lean mass together with ethnicity in ANCOVA resulted in an attenuation of the contribution of ethnicity to BMD at all sites, particularly at the lumbar spine. In

males, lean mass did not explain any of the differences observed in BMD between the ethnic groups possibly because lean mass was not different between Black African and Indian males. Genetic factors, which have been shown to be responsible for 50-80% of the variance at any age or in any population group (428) were not investigated in this study.

The association between fat mass and BMD is contentious with some studies showing positive associations (429, 430), whilst others have reported negative associations (431, 432). This may be related to ethnic, age or gender differences between the populations studied. In this study, the effect of fat mass differed by ethnic group, being positively associated with hip, neck and lumbar BMD in the Black African group, but inversely associated with subtotal BMD in the Indian group. Fat may increase BMD by mechanical loading and the well as the conversion of androgens to oestrogens in adipose tissue (433) and reduce BMD via the effect of proinflammatory cytokines such as IL-6 and TNF-α, which are increased in obesity (37), or through adiponectin levels which fall with increasing obesity. Ethnic differences in adipokines levels have been reported (434) and further investigation is warranted to determine if this may explain the different associations between BMD and fat mass observed in different ethnic groups in the current study.

After adjusting for a number of covariates, visceral adiposity was negatively associated with lumbar BMD in the Indian group and subtotal BMD in the Black African group, while sub-cutaneous adiposity was not associated with BMD at any of the sites. Similar negative relationships between visceral adiposity and bone parameters have been reported in young women (435), young men (436) and obese men (437). However despite several studies consistently showing an inverse association between visceral adiposity and BMD, the associations between sub-cutaneous adiposity and BMD less consistent, with absent (435, 438) positive (412) and negative (424) correlations all being reported. These inconsistent findings may be due to differences in body fat distribution between different populations, or in bone parameters studied. The consistent inverse relationship between visceral adiposity and BMD across studies, suggests that it is not influenced to the same degree as sub-cutaneous adiposity by various confounders (439).

Only 12% of the variance in lumbar BMD in both African and Indian subjects was explained by the variables in the multiple regression models. This is much lower

than the variance explained at other skeletal sites. Our results are similar to those of Chantler et al, who showed that body composition, socioeconomic factors and lifestyle only explained 22% of the variance at the lumbar spine compared to 33-42% variance at other sites (407). The lumbar spine consists predominantly of trabecular bone, which is not influenced as much by mechanical forces as it is by the metabolic and hormonal mileau. It is possible that adipokines or hormones play a more important role in determining BMD at the lumbar spine than the variables that we have measured in this study.

As far as we are aware this is the first study to report on visceral adiposity in Black African males, as well as the differences in body composition between Black African males and females from this region. The Black African women had more visceral adiposity than the Indian women, despite similar levels of total body fat. A previous study has shown the reverse; however that investigation had a much lower N than the current study (9). Male Black African subjects had less visceral fat than their Indian counterparts due to the much greater level of total body fat in the latter group. The association of regional fat distribution with disease outcomes in black South African males and females requires further investigation.

This is also the first study to report on subcutaneous adipose tissue differences among the two ethnic groups. A number of studies have shown that Indian females have greater subcutaneous adiposity compared to Indian males (440) similar to our findings. Two compartments of subcutaneous tissue have been described, superficial and deep subcutaneous, distinct anatomically and morphologically (441, 442). Women shown to have more superficial fat and men greater deep subcutaneous fat. These differences merit investigation in Black African populations to further our understanding of ethnic and gender disparities in health.

Serum 25(OH)D levels contributed weakly to ethnic differences at all BMD sites in males but not in females. This may be a reflection of the relatively high 25(OH)D levels in African males (72.7nmol/L (IQR: 51.1, 94.1). Previous reports suggest that 25(OH)D above a threshold of 50nmol/L(418, 443) or 75nmol/L(444) are associated with higher BMD, while other studies in which mean 25(O)D concentrations were lower than 75nmol/L have shown no association between BMD and 25(OH)D (148, 445, 446).

Within the current study the BMD at all skeletal sites, except for the lumbar spine, correlated negatively with PTH in the Indian subjects whilst in the Black African group PTH correlated negatively with hip BMD only. Previous studies performed in Indian subjects have yielded similar results to ours, with 25(OH)D demonstrating no correlation to BMD but PTH showing an inverse association with BMD (419, 447). Adult African-Americans are reported to show skeletal resistance to PTH (448) which may explain the lower incidence of osteoporotic fractures in black subjects. Whether this is true of the Black African population in South Africa remains to be confirmed.

A major strength of this study is the bi-ethnic sample of males and females with quantitative measures of BMD using dual energy x-ray absorptiometry. However this was a cross sectional study and while linear regression models can be used to explore relationships between predictors and outcomes they do not prove a cause-effect relationship. As we did not have data on the menopausal status of the female subjects age was categorized as < 50 years or > 50 years. Data from our centre suggests that African females attain menopause at a mean age of close to 50 years (Jaff et al., unpublished data). We have not measured sex hormones or serum adipokines, which are related to fat mass and may have contributed to the BMD at the different skeletal sites or on physical activity, which may also contribute to BMD. A further limitation of this study is that ultrasound was used to measure visceral and subcutaneous adiposity and this method is not the current gold standard technique. However previous studies, including one performed in black South African subjects (23), have reported ultrasound as being a precise and reliable method of measurement (339, 449).

5.5 CONCLUSIONS

In conclusion, we have shown that whole body and site-specific BMD is significantly higher in Black African males and females compared to Indians of a similar age which may explain the differential fracture risk between these ethnic groups (404). We have shown that lean mass significantly contributed to BMD at all sites in both ethnic groups and visceral adiposity contributed negatively and differentially to BMD sites in the two ethnic groups. As significantly less of the variance in lumbar BMD, in both ethnic groups, could be explained compared to the other sites future studies should endeavour to determine what other factors contribute to BMD at this site in these population groups. Public health

interventions aimed at improving bone health should include efforts to increase lean mass and reduce abdominal obesity.

CHAPTER 6

6. DISCUSSION AND CONCLUSIONS

This chapter summarises the results of the study, highlighting key points in table 6.1.

6.1 SUMMARY OF FINDINGS

The hypothesis to be tested in this thesis was that ethnic differences in vitamin D status contribute to ethnic differences in metabolic syndrome or its components and to ethnic differences in BMD.

Table 6.1: Consolidated Findings

Objectives	Chapter	Evidence
1. Describe the prevalence of 25(OH)D deficiency in Black Africans and Indians in Johannesburg	3	Using a cut off of 25(OH)D of <30nmol/L to define deficiency the prevalence of deficiency was 28.5% in Indians as opposed to 5.1% in Black Africans (p<0.0001).
2. Identify predictors of 25(OH)D serum levels	3	Parathyroid hormone (PTH) was negatively associated with 25(OH)D while season and sunshine exposure were positive predictors explaining 16% of the variance (p<0.0001) in Black Africans. In Indians, PTH was negatively associated with 25(OH)D while male gender, season and calcium supplementation were positive predictors and explained 17% of the variance of 25(OH)D (P<0.0001).
3. Examine the relationship between body fat distribution and 25(OH)D levels	3	In multivariate regression analysis, neither total body fat nor body fat distribution was predictive of 25(OH)D in either group.
4. Test the association of 25(OH)D or PTH with Met S in Black Africans and Indians in Johannesburg	4	Met S was diagnosed in 29% of the Black African and 46% of the Indian subjects (p<0.0001). Subjects with Met S had higher PTH than those without Met S, (p<0.0001), whilst 25(OH)D levels were not significantly different (p=0.50).
Determine if 25(OH)D or PTH explained ethnic differences in the prevalence of Met S and its individual components.	4	In multivariate analysis, 25(OH)D was not associated with any components of the Met S however PTH was shown to be positively associated with systolic (p=0.018) and diastolic (p=0.005) blood pressures and waist circumference (p<0.0001) and negatively associated with HOMA (p=0.0008) levels. Logistic regression analysis showed that Indian ethnicity (OR 2.24; 95% CIs 1.57, 3.18; p<0.0001) and raised PTH (OR 2.48; 95% CIs 1.01, 6.08; p=0.04; adjusted for 25(OH)D) produced an increased risk of Met S but 25(OH)D did not (OR 1.25; 95% CI 0.67, 2.24; p=0.48)

Table 6.1 continued

Objectives	Chapter	Evidence
5. Compare the body composition, including BMD, of Black African black men and women to Indian men and women.	5	Black African females were significantly heavier and had a greater BMI than Indian females (p=0.001), but waist circumference was not significantly different (p=0.9), whilst Black African males weighed less (p<0.0001), had a lower BMI (p=0.002) and a lower waist circumference than Indian males (p<0.0001). VAT was significantly higher in Black African females and Indian males compared to their same sex counterparts, and SCAT was significantly lower in Black African males compared to Indian males. All BMD measures were significantly higher in Black African males and females compared to their Indian counterparts. These differences remained significant after adjusting for age, height, weight and site-specific bone area.
6. Understand how body composition contributes to BMD at various skeletal sites in both ethnic groups, and	5	Whole body lean mass positively associated with BMD at all sites in both ethnic groups (p<0.001 for all), and partially explained the higher BMD in Black African females compared to Indian females. Whole body fat mass correlated positively with lumbar BMD in Black African (p=0.001) and inversely with sub total BMD in Indian subjects (p<0.0001). Visceral adiposity correlated inversely with sub total BMD in the Black African (p=0.037) and with lumbar BMD in the Indian group (p=0.005).
7. Determine whether the effects of body composition on BMD at various skeletal sites are mediated via PTH or 25(OH)D.	5	No association was found between serum 25(OH)D and BMD. PTH was inversely associated with hip BMD in the Black African group (p=0.01) and with sub total (p=0.002), hip (p=0.001) and femoral BMD (p<0.0001) in the Indian group.

Key: BMD=bone mineral density, STBMD= subtotal BMD, HBMD= hip BMD, LSBMD= lumbar spine BMD, VAT=visceral adipose tissue, Met S=metabolic syndrome

In summary this study has highlighted a number of points:

1) The high prevalence of 25(OH)D deficiency and insufficiency in the Indian population, particularly in women
2) The association of PTH with Met S in both ethnic groups
3) Differences in body composition among ethnic groups

In view of the unexpected findings of the lack of association of vitamin D with the Met S and the contribution of PTH to Met S an alternative hypothesis is that PTH increases risk of Met S

6.1.1 Vitamin D Deficiency and Insufficiency

Prevalence

This is the first large study from South Africa to report on the prevalence of vitamin D deficiency in Black African and Indian populations. It highlighted the high prevalence of 25(OH)D deficiency in the Indian population, with the Black African population being relatively 25(OH)D replete. 25(OH)D levels in South African women have been shown to be above 50nmol/L, not only in this study but also by Hamill et al (450) in HIV positive women from the Johannesburg area and by Kruger et al (300) in urban women from the North West province. There appear to be no data on Black African men or on the Indian population resident in this area. It is interesting that the prevalence of vitamin D deficiency as defined by a serum 25(OH)D level below 30nmol/L was 28.6% in Indians vs. 5.1% in Africans. While it is difficult to compare data across studies due to differences in cut offs used to define vitamin D deficiency, the prevalence of vitamin D deficiency appears to be high in Indians across the globe (90, 451), including those who living in sunny countries (180, 452). A prospective cohort study from the Greater Manchester area in the UK noted that lifestyle differences including exposure to sunshine lead to lower 25(OH)D concentrations in Indians in comparison to Caucasians (362). While this thesis did not determine the reason for the differences observed, it is possible that these arose largely as a result of cultural practices.

Seasonal Variation and Sunshine Exposure

Seasonal variations in 25(OH)D levels were noted in all groups, with the lowest levels of 25(OH)D for winter and spring collections. The highest levels were noted in summer and early autumn which is consistent with other studies worldwide. However, it is not known whether serum levels obtained at a single time point are reflective of long term 25(OH)D levels and there are limited data on tracking vitamin D levels. Jorde et al tracked serum 25(OH)D over 14 years in a Norwegian cohort and noted a correlation of between 0.42 and 0.52 (453), concluding that a single measurement was sufficient to predict future outcomes. These studies should be repeated to test whether this is true of other populations.
As discussed in chapter 3, sun exposure scores were similar for both ethnic groups, but differed by gender within groups.

6.1.2 Metabolic Syndrome, 25(OH)D and PTH

Prevalence

We noted a Met S prevalence of 29% in Africans compared with 49% in Indians using the harmonized definition of the Met S. While nationally representative data are lacking from South Asian countries, a study conducted in Karachi, Pakistan showed a prevalence of 34.8-49% according to the International Diabetes Federation (IDF) and the National Cholesterol Education Program Treatment Panel III (NCEP ATP III) criteria (454), and data from India suggest that up to one third of the urban population has the Met S (31, 455). Similarly, there are limited data on the prevalence of the Met S in South African populations. Using the harmonized definition of the Met S, Crowther et al noted a prevalence of 42.1% in urban women (331), while a study conducted in a rural population of men and women gave a prevalence of 22.1% (28). The high prevalence of the Met S in our study as well as in the study by Crowther et al was due mainly to abdominal obesity.

While 25(OH)D has been associated with individual components of the Met S and with the Met S itself, this was not true of the group we studied. As described in the introduction, the results of observational studies on the association of 25(OH)D with the Met S often yield conflicting results, but generally suggest that there is an association between 25(OH)D and blood glucose levels. There are several possible reasons for these inconsistencies that have been discussed in the introduction, including failure to adjust for BMI. However it is possible that there is a threshold 25(OH)D level below which metabolic effects are seen. In a single longitudinal study of high risk individuals followed up over five years, Lim et al noted that the hazards ratio for T2DM was greatest for those with concentrations <25nmol/L compared to those above 50nmol/L (271). It is also possible that the differences observed may be as a result of low levels of free or bioavailable 25(OH)D resulting from variation in the affinity of vitamin D binding protein (DBP) for 25(OH)D. DBP is the primary vitamin D carrier protein, binding 85-90% of total circulating 25(OH)D (456). Less than 1% of total 25(OH)D is in the free form. DBP appears to inhibit some of the actions of vitamin D, because the bound fraction may not be bound to target cells (457). Genetic polymorphisms in DBP produce variants that differ in their affinity for 25(OH)D and the prevalence of these polymorphisms varies across populations (458, 459). All assays that are currently in use for diagnostic purposes measure total 25(OH)D without distinguishing free or bioavailable 25(OH) from the bound form.

PTH and the Met S

We have shown that PTH is higher in subjects with the Met S. This is in keeping with a number of other studies in adults (255, 382). PTH has also been shown to be an independent predictor of insulin resistance and inflammation in adolescents (460). Even a modest elevation of PTH is associated with increased risk of death from cardiovascular causes (461, 462). In addition observational studies link PTH to a higher risk of diabetes, hypertension, hyperlipidaemia and cardiovascular disease(390, 463, 464). PTH levels are inversely related to 25(OH)D levels, rise in chronic renal disease and with increased obesity (148, 465, 466). There are a number of possible reasons for the rise in PTH that occurs with obesity, one being the inverse relationship of 25(OH)D with obesity. It has been postulated that this is due to sequestration of vitamin D in fat (316, 467), reduced physical activity and time spent in the sun (468). Another candidate for the positive association between fat mass and PTH is leptin, levels of which are increased with obesity. Administration of leptin to deficient mice (*ob/ob*) greatly increases PTH suggesting that leptin is a PTH secretagogue (469). Leptin has been shown to be a predictor of PTH and FGF 23 (397).The resultant increased PTH may then affect cardiovascular function by altered vascular reactivity possibly by interfering with endothelial function (470). Secondly, PTH may increase cardiovascular risk by increasing blood pressure. A positive association between PTH and blood pressure has been reported not just in our study, but by other investigators (471-473). Supportive clinical evidence comes is shown in studies from patients with primary hyperparathyroidism who have enhanced responsiveness to pressor agents such as angiotensin II (474). In addition, increased PTH levels stimulate renin and aldosterone synthesis, both of which are important mediators in arterial hypertension, by increasing intracellular calcium levels (475). A third mechanism whereby PTH can increase the risk of cardiovascular disease is via altered insulin sensitivity and secretion perhaps via calcium (476). Therefore an alternative hypothesis to the 25(OH)D theory is that PTH may not be an innocent bystander but may be directly involved in the pathogenesis of cardiovascular disease (see figure 6.1).

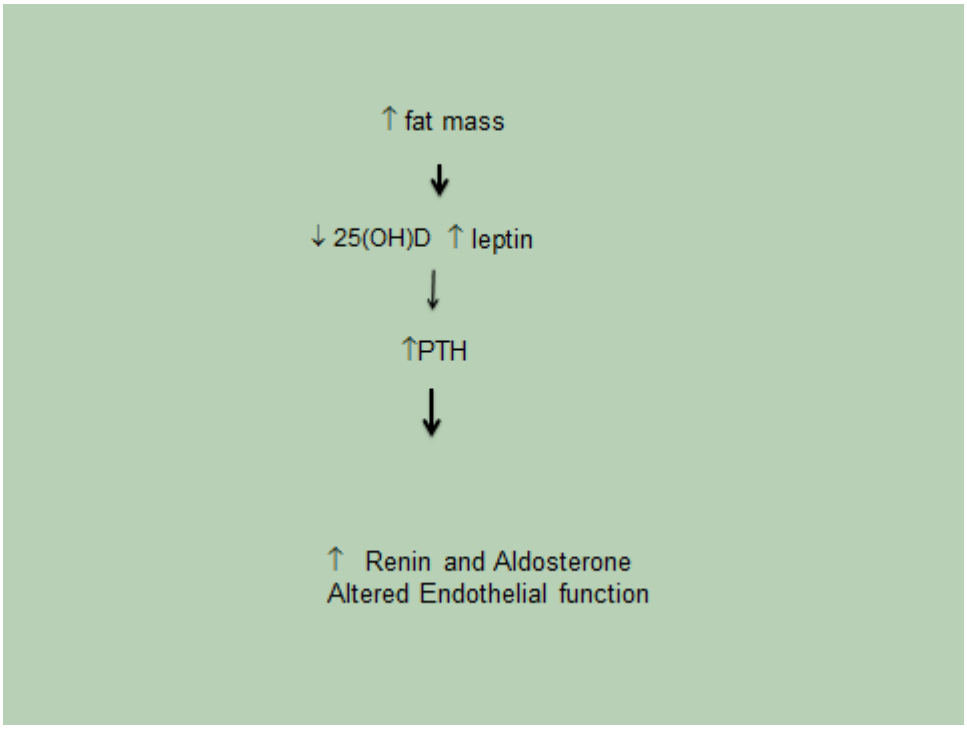


Figure 6.1: The Role of PTH in the Met S

6.1.3 The Role of Body Composition, 25(OH)D and PTH on Bone Mineral Density in Black Africans and Indians

Since both PTH and 25(OH)D are important in maintenance of bone health which is also affected by lean mass and fat mass the third paper examined differences in body composition between Black Africans and Indians as well as the effect of body composition, 25(OH)D and PTH on bone mineral density.

Osteoporosis is characterized by compromised bone density that predisposes affected individuals to increased fracture risk (477). Extensive epidemiological data has shown that high BMI is associated with increased bone mass, and that reductions in body weight may cause bone loss (478, 479). However there are also data suggesting that obesity may have a detrimental effect on bone. For example obesity and osteoporosis co-exist in subjects with T2DM (480). Apart from the effect on BMD in older people, it has also been associated with increased fracture risk in young adults and children (481, 482). There are limited data from Africa on BMD or its predictors. A systematic review of worldwide hip fracture incidence and probability of fracture (483) reported South Africa as being a low risk area referencing a 1968 study as the reference (404). The table below

summarizes the findings of some studies that have compared BMD in Africans or Indians to BMD in Caucasians.

Table 6.2: Comparisons of Bone Mineral Density of Africans and Indians with Caucasians as the reference

Study population (reference)	Bone Site	N	Results
US adults NHANES NHB and Mexican Americans ,(484)	LS	7912	LS BMD >> in NHB compared to Caucasians
#Osteoporosis Fractures in men Study(US adults, Afro-Caribbean, Hong Kong Chinese and South Korean (485)	FN H LS	4074 Caucasian, 627 African American,	BMD at all three sites >> in African-American compared to Caucasians
South African Black African and Caucasian women (407)	FN H LS		BMD FN and H >> in Black African compared to Caucasians but << at LS
Indians males and females compared to Caucasian database for densitometer (425)	FN H S FT IT	1137 Indians	Indians had << BMD at all sites
## Indian females compared to reference values for US Caucasian females (486)	H LS	2034 Indian women	Indians had lower BMD of LS compared with US Caucasian females

Key: NHB=Non-Hispanic Blacks, LS =Lumbar Spine, FN=Femoral Neck, H=Hip, LS=Lumbar Spine, FT=Femoral Trochanter, IT-Intertrochanteric

African included US and Tobago African American.

Database established from 1472 white women for spine data and 1487 white women for femur

There are no published data on BMD in Black African men and as far as we are aware our study is the first report on BMD in Black South African males. In chapter five we noted that Black African subjects had greater BMD than Indian subjects. In both ethnic groups lean mass was the major contributor to BMD. The results of this thesis show that there are racial differences in total lean mass for subjects with similar BMI. We showed that lean mass was significantly higher in Black African women compared with Indian women, while BMI was not significantly different for these groups. Although BMI was significantly higher in Indian men compared to Black African men, lean mass did not differ. Black women have been shown to have greater lean mass than Caucasian women (487, 488) while Chinese and

South Asian men and women have been shown to have less lean mass than Caucasians (433).

Another observation was that while total fat mass was significantly lower in Black African men compared to Black African women, this was largely due to greater subcutaneous adiposity in African women as visceral adiposity was not significantly different. We reported greater visceral adiposity among African women compared to Indian women. This is contrary to a previous study from our center by Waisberg et al (402), with the main differences being that in our study visceral adiposity was measured by ultrasound which is not the gold standard, whereas CT was used in the other study and in Waisberg's study the subject numbers were small. In spite of greater visceral adiposity in African women, we showed that HOMA-IR was greater in Indian subjects compared with African subjects (chapter three).

There are data to suggest that factors other than the amount of adipose tissue may be involved in disease pathogenesis. Up to 25% of morbidly obese subjects are insulin sensitive (489). The non-adipose contributors to insulin sensitivity are not well defined but may include differences in inflammatory cytokines, adipokine secretion, AMP kinase activity and oxidative stress (490) and amount of lean mass (491). Lean mass, which is predominantly skeletal muscle, is a primary target of insulin action and increases are associated with improved insulin sensitivity (492). While the adverse health effects of excess fat are well established, the impact of low lean mass is only beginning to be considered. Reduced lean mass is associated with reduced bone mineral density, poorer clinical outcomes (493) and with cardiovascular risk (494). Therefore, although much attention has been paid to differences in fat mass, variability in lean mass and lean mass distribution may be equally important.

PTH was inversely and differentially associated with BMD sites for both ethnic groups, while 25(OH)D was not associated with BMD at any site in either group. However, in our study it contributed minimally to differences in BMD between African and Indian males.

The relationship between serum 25(OH)D and BMD has been shown to differ between black and Caucasian races (447, 495-497). Although African-Americans have lower levels of 25(OH)D than do Caucasian Americans, they have a lower

prevalence of osteoporosis (498). Secondary hyperparathyroidism has been proposed as the mechanism whereby hypovitaminosis D causes low BMD.

PTH increases mainly cortical, but also trabecular bone turnover (495, 499). The relationship between 25(OH)D, PTH and BMD has been shown to differ by race in studies from the United States (148). Whether this is true of other populations in southern Africa remains to be determined.

6.2 THEORETICAL, LOCAL AND NATIONAL RELEVANCE

We have shown a very high prevalence of the Met S in Black Africans and Indians, across a wide age spectrum, using the harmonized definition of the Met S. This is an indicator of cardiovascular risk and contributes to the already existing body of knowledge on the scale of NCDs in South Africa. A recently published South African survey noted that the prevalence of NCDs based on self-report was 51.8% in people over the age of 50 (500). It can be attributed to a number of factors including lifestyle, socio-economic status, ethnicity and urbanization.

Furthermore, while we have shown that the prevalence of vitamin D deficiency was high in the Indian population, it did not predict the Met S in either population group. PTH was predictive of the Met S in both groups, and as discussed, PTH is influenced, possibly directly by fat. More than one in two South African women and one in three adult men are either overweight or obese (9). We also noted significant differences in body composition between the groups which may contribute to disease risk.

In the 1980s Barker proposed that many nutritional events that occur in intrauterine life and early in infancy influence the development of adult diseases (501). While we did not find an association of 25(OH)D with either the Met S or BMD, we do not know how vitamin D deficiency in the intrauterine environment contributes to increased cardiovascular disease. Incorporating this hypothesis, the interrelationships illustrated in Figure 1.4 (chapter 1) can be modified to give a conceptual framework as illustrated in Figure 6.2. One addition, worth considering is the addition of PTH into the model.

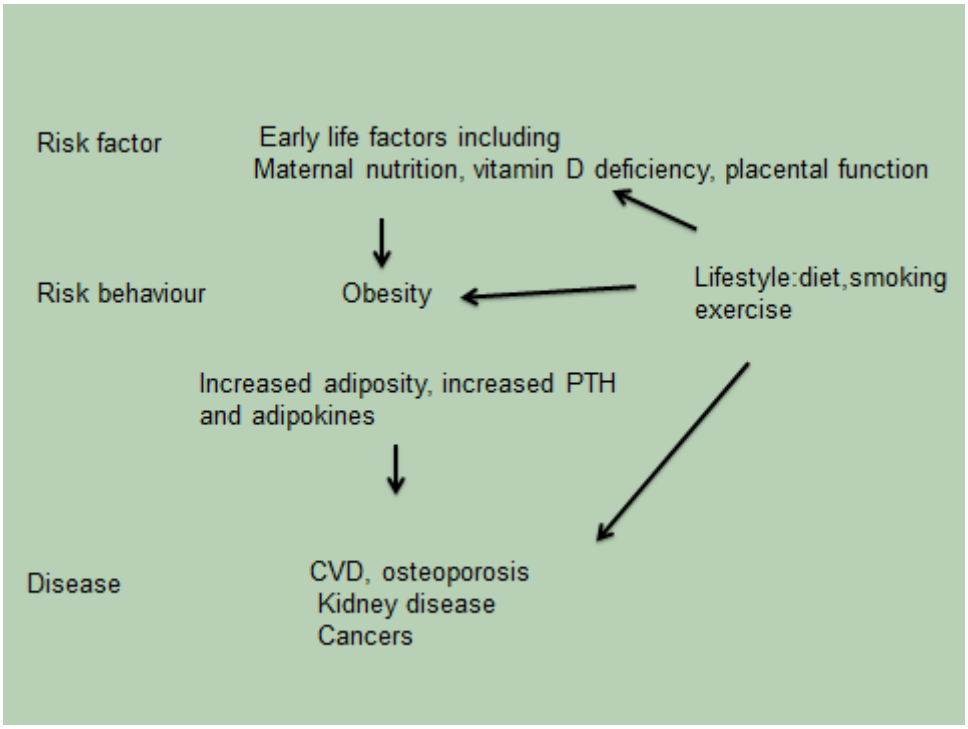


Figure 6.2: Possible Interrelationships between PTH, Traditional Risk Factors and Non-Communicable Diseases

6.3 AREAS OF FUTURE RESEARCH

As this was a cross sectional study the results presented cannot prove causality, and longitudinal studies would be useful to further investigate some of the associations noted in the thesis.

The Relationship of PTH to Cardiovascular Risk and Body Fat in Black African and Indian Populations

PTH has been shown to be positively related to BMI and body fat. It is not known if both visceral and subcutaneous adiposity are involved in this association or whether adipose tissue secretes PTH, or if all secreted PTH arises from the parathyroid gland.

It would also be necessary to carry out a longitudinal study to investigate how PTH and 25(OH)D change over time and how these changes affect disease risk. The PTH/25(OH)D threshold varies with ethnicity and has not been determined for Africans or Indians. We would like to determine this threshold in a larger group of subjects.

The Relationship between DBP, Total 25(OH)D and Free 25(OH)D

There are several possible reasons for the lack of association of 25(OH)D with the Met S. One being that it is inappropriate to define deficiency at levels <30 nmol/L of total 25(OH)D in our populations. The free hormone hypothesis postulates that only hormones liberated from binding proteins enter the cells and produce biological action (502). 25(OH)D and 1,25(OH)D circulate bound to DBP and albumin with less than one percent in the free form (503). Recent data from the United States has shown that while black subjects had lower total 25(OH)D than whites they had similar concentrations of bioavailable 25(OH)D (504). Polymorphisms in DBP contribute to variation in free 25(OH)D. At present there are no data on DBP concentrations or polymorphisms in Black African or Indian populations. It is important to determine what the concentrations of DBP are in these populations and how these relate to free 25(OH)D levels and to PTH levels. It is also important to determine what the PTH/ bioavailable 25(OH)D threshold is in these populations are, which may provide an indication of optimal 25(OH)D levels in these groups. Identifying those individuals with a true deficit will allow for interventions for those individuals most likely to benefit.

The Effect of Vitamin D Deficiency on Pregnancy and Child Outcomes

Another area for future research is the effect that low levels of 25(OH)D may have on pregnancy and on neonatal and infant outcomes. During pregnancy, maternal 25(OH)D levels may decrease, particularly during the third trimester (505). At birth, cord blood 25(OH)D levels are directly correlated with maternal levels (506). Since Indian women are clearly an at-risk population, it would be important to follow up a cohort of women to investigate levels seen in the two groups and the effects, if any, on pregnancy and neonatal outcomes. A recent systematic review and meta-analysis of observational studies showed that vitamin D insufficiency was associated with an increased risk of gestational diabetes, pre-eclampsia, small for gestational age babies and bacterial vaginosis (507). In nested case control studies mid-gestation 25(OH)D levels of <50nmol/L were associated with almost 4 fold odds of severe pre-eclampsia (508) and levels <37.5nmol/L with a 5 fold risk of severe pre-eclampsia (509). Risk factors for pre-eclampsia include increased BMI, diabetes, chronic renal disease and hypertension as well as multiple pregnancies (510). Several of the above are also risk factors for 25(OH)D deficiency. Additional concerns are the effects of maternal 25(OH)D deficiency on the skeleton of the foetus.

6.4 LIMITATIONS

Subjects were recruited via caregivers of the Birth to Twenty cohort, and though the caregivers were randomly selected, there may be bias which would affect the generalizability of the findings beyond the study population. Furthermore, the Birth to Twenty cohort represents a group of subjects based in Johannesburg. The caregivers represent long-standing residents of Johannesburg and do not include recent migrants and may not be representative of contemporary Johannesburg. It can be argued that Johannesburg is not representative of Black African and Indian populations living in other parts of the country and as such the thesis findings represent specific subpopulations within South Africa. Whether these data can be generalizable to other areas in the country is speculative. However the research highlights the importance of conducting similar studies in different South African settings and population groups.

This study was cross-sectional and as such does not prove causality for any of the associations described. All biochemical measurements were performed at a single time point and may not necessarily be indicative of long-term status. For assessment of predictors of 25(OH)D deficiency, it would have been ideal to have used a dosimeter to assess sunshine exposure. The sunshine exposure score that we used has been used in a number of studies but has not been validated (341). We carried out a limited assessment of vitamin D and calcium intake from diet, and calcium and vitamin D supplementation was determined as a 'yes' or 'no' response and was not quantified. We have suggested that wearing of the veil may be the cause for the lower vitamin D levels in Indian women however we did not assess how many Indian women used the veil. We also did not assess how many were vegetarians. Studies report contradictory results on the relationship between a strict vegetarian diet and vitamin D deficiency. Low serum vitamin D levels and low bone mass have been reported in some vegan groups who do not take vitamin D supplements (511). On the other hand Chan *et al* in a comparison of vegetarians, partial vegetarians and non-vegetarians noted that 25(OH)D levels were not associated with vegetarian status (512)

HIV-infection was based on self-report. This may not yield reliable results due to stigmatization or not being tested. In addition the type of ART that subjects took was not recorded. Hamill *et al* noted that there was no difference in vitamin D or BMD between South African women eligible to receive anti-retrovirals, and those infected but with preserved CD4 counts as well as with healthy

controls(450).There are reports that the use of Tenofovir is associated with significant loss of BMD (513)

This study was not primarily designed to look at reasons for differences in BMD. Socio- economic status and lifestyle (407) have been shown to contribute to variance in BMD. We assessed education as a proxy for socio-economic status and it did not correlate with BMD. It would have been preferable to use a questionnaire that determined employment activity, housing density and physical activity as these may have provided some data on reasons for differences in BMD between the different ethnic groups. Other important contributors to BMD include sex steroids and these were not measured. Visceral adiposity and subcutaneous adiposity were determined by ultrasound assessment of thickness and not CT scan or MRI. However, the ultrasound measurement of body fat depots has been validated against a gold standard (339).

6.5 CONCLUSIONS

This thesis set out to determine the prevalence of vitamin D deficiency in Black Africans and Indians living in Johannesburg. It also was designed to determine if 25(OH)D or PTH contributed to cardiovascular disease in both populations. The final aim was to determine differences in body composition and the role of 25(OH)D and PTH in BMD in both populations.

This thesis provided evidence for an association of PTH with the Met S via its positive association with blood pressure and waist circumference, and an inverse association of PTH with BMD at a number of skeletal sites in both Black Africans and Indians. Longitudinal studies are needed to confirm these findings and to explore the relationship between fat and PTH.

The data from this thesis do not support the notion that either bone mineral density or cardiovascular risk is compromised by 25(OH)D levels. Overall it is important that we identify the vitamin D levels associated with best health in our population groups. Until we have the results of studies designed to determine the thresholds of vitamin D that are associated with optimal health in our populations it is inappropriate to define 25(OH)D levels of less than 50nmol/L as insufficient, as this implies that supplementation is required and we have no clinical evidence from South Africa that this cut-point is appropriate to treat. .

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APPENDIX 1

- **ETHICAL CLEARANCE**

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Jaya George

CLEARANCE CERTIFICATE	**M10411**
PROJECT	Determination of Vitamin D Status in Local population Groups in gauteng
INVESTIGATORS	Dr Jaya George.
DEPARTMENT	Department of Chemical Pathology
DATE CONSIDERED	30/04/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 28/05/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX 2

- **FOOD FREQUENCY QUESTIONNAIRE (FFQ) 2011**

Study of Vitamin D and Cardiometabolic risk

FOOD FREQUENCY QUESTIONNAIRE (FFQ) 2011

DATE: **Day** ☐☐ **Month** ☐☐ **Year** ☐☐☐☐

☐☐☐☐☐☐☐

BTT ID NUMBER: ☐☐☐

DCR ID NUMBER:

FOOD HABITS

1. Are you on a special diet that has been prescribed for you e.g. by a doctor or one that you have adopted from someone e.g. a TV star/magazine?

YES	1
NO	0

2. If NO, go to question 4.
   If YES, describe what kind of diet you are on and where you got the diet from?

   _____

3. How long have you been on that diet? _____ months/years

4. Do you currently take any vitamin and mineral supplements?

YES	1
NO	0

IF YES, what do you take?

	Name of product	Amount/day (or how many tablets)
Vitamins/vitamins and minerals		
Tonics		
Vitamin D		
Calcium		
Body building preparations		
Dietary fibre supplement		
Other: specify		

5. Which meals do you skip almost on a daily basis?

Breakfast	1
Lunch	2
Evening meal	3
None	4

6. Is salt added to your food while it is being cooked?

Always	1
Sometimes	2
Never	3
Don't know	4

7. Do you add salt to your food before you eat it?

YES	1
NO	0

8. If yes, how much salt do you add to your food each day?

¼ teaspoon	1
½ teaspoon	2
¾ teaspoon	3
1 teaspoon	4
Other specify:	5

9. Do you add Aromat to your food before you eat it?

YES	1
NO	0

10. If yes, how much Aromat do you add to your food each day?

¼ teaspoon	1
½ teaspoon	2
¾ teaspoon	3
1 teaspoon	4
Other specify:	5

11. There are some factors which influence the choice of foods we eat. Which of the following statements are true for you?

	Strongly agree	**Agree**	**Disagree**	**Strongly Disagree**
I choose to eat certain foods because they taste good	1	2	3	4
The food I eat depends on whether it is expensive	1	2	3	4
I choose to eat certain foods because it looks good	1	2	3	4
The food I choose to eat differs according to my mood (i.e. happy/sad)	1	2	3	4
My hunger level determines what type of food I eat.	1	2	3	4
I choose foods which are not time consuming to prepare	1	2	3	4
I consider whether a food is good for my health before eating the food.	1	2	3	4

12. Do you ever eat outside the home e.g. at fast food shops such as Nandos, KFC and Steers?

YES	1
NO	0

13. If YES, in an average month how often do you eat at the following places?

	Frequency of visits		
	Times/week	**Times/month**	**Rarely/never**
Nandos			
Spur			
Macdonalds			
Steers			
KFC			
Chicken Licken			
Debonaire's Pizza			
Romans			
Anat			
Wimpy			
Something fishy			
Fontana			
Chinese takeaway			
Other restaurants/takeaways			
(Quarters from tuck shop)			

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	DAIRY-BLUE									
	1.Tea	Ordinary		4038						
		Herbal		4053						
		Rooibos		4054						
	1. Sugar in tea			3989						
	2. Milk in tea	Full cream		2718						
		Low fat 2%		2772						
		Skim fat free		2775						
		Other								
	1. Coffee			4037						
	2. Milk in coffee	Full cream		2718						
		Low fat 2%		2772						
		Skim fat free		2775						
		Other								
	2. Sugar in coffee			3989						
	2. Milk as a drink	Full cream		2718						
		Low fat 2%		2772						
		Skim fat free		2775						
	3. Buttermilk/maas	Buttermilk		2713						
		Maas		2787						
	4. Milk drinks, flavoured									
X	**5. Yoghurt**	Fruit, LF, sweetnd		2732						
		Plain LF		2734						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	3. Breakfast cereals									
	2. Milk added to cereal									
	1. Sugar added to cereal			3989						
	9. Ice cream	Full cream		3519						
		Low fat		4325						
	9. Ice lollies			3982						
	ASK ABOUT TYPE OF BREAD,THICKNESS OF SLICES AND THE SPREAD									
	1. Bread/rolls	White		3210						
		Brown		3211						
		Whole wheat		3212						
		Traditional		3210						
		Roti								
	Spread? Y / N	**Brand name**	*Do you use spread every day on your bread?*							
	1. Brick margarine	HM		3484						
	1. Tub margarine	PUM		3496						
	1. other tub e.g. floro			3521						
	1. Butter			3479						
	Cheese spread	WM		2730						
	Fish paste			3109						
	Honey/syrup			3988						
	Jam			3985						
circle	**Marmite/ Bovril**			4030/ 4029						
	Sandwich spread			3522						
	Peanut butter			3485						
	Chocolate spread			9999						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	Cold meats	Ham		2969						
		Polony		2919						
		Salami		2948						
	6. Cottage cheese	Low fat		2729						
		Full fat		2759						
X	**7. Cheddar**			2722						
	7. Gouda			2723						
	7. Other cheese									
	8. Cheese wedges			2728						
	2. Fat cakes			3257						
	Any topping?									
(Red)	**QUARTER**	*Tick toppings:*								
	White bread			3210	¼ loaf = 225					
	Fried Chips			3740	1 portion = 90					
	Cheese			2722	___ slices x 20 =					
	Polony / French			2919	___ slices x 15=					
	Vienna (Russian)			2936	___ unit x 40 =					
	Fried egg			2869	___ medium x 45 =					
	Atchar			3117	___ Tsp x 40 =					
	Tomato sauce			3169	___Tsp x 25 =					
Other	**Roti**									
Other										
	Mashed potato			3876	¼ cup = 62.5					
	Mince meat, regular			2987						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	4. Maize porridge stiff			3400						
	4. Maize porridge soft									
	2. Milk on soft porridge									
	1. Sugar on soft porridge			3989						
	1. Fat on soft porridge									
	4. Mabele/maltabella stiff			3241						
	4. Mabele soft									
	2. Milk on Mabele									
	1. Sugar on Mabele			3989						
	1. Fat on Mabele									
	4. Oats			3239						
	2. Milk on Oats									
	1. Sugar on Oats			3989						
	4. Maize and pumpkin porridge									
	5. Pasta/Pasta dishes									
	Remember to ask about added cheese! Grated?									
	6. Spaghetti Bolognaise	Regular mince		3260						
	6. Macaroni and cheese	WH, HM		3301						
	6. Lasagne	Regular		3261						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	7. Rice	White		3247						
		Brown		3315						
		With fat?								
	Biriyani									
	7. Samp/mealie rice	With fat?								
	7. Samp and beans	With fat?		3402						
	7. Wheat rice			3249						
X	**8. Pizza**	Vegetable topping								
		Meat topping								
		Meat and vegetables								
	8. Savoury tart									
X	**EGGS-YELLOW**									
	Boiled/poached			2867						
	Fried	HM		2877						
		PUM		2878						
		Sun oil		2869						
tick	**Scrambled**	LFM, PUM		2888						
		LFM, Sun oil		2889						
		WM, HM		2890						
		WM, Sun oil		2873						
		WM		2872						
	Omelette (tick scrambled and add topping here)									

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	FRUITS-ORANGE									
X	**1. Apples**			4222						
X	**2. Bananas**	Fresh		3540						
		Fried		3611						
	3. Berries	Fresh								
		Candied								
X	**4. Figs/prickly pears and**	Fresh								
	Coconuts, dates	Candied								
	5. Fruit salad	Fresh								
		Sugar added?								
		Canned juice		3667						
		Canned syrup		3580						
X	**6. Grapes**			3550						
X	**7. Guavas**	Fresh		3551						
		Canned juice		3628						
		Canned syrup		3553						
X	**8. Mango**	Fresh		3556						
		Canned syrup		3633						
	8. Pawpaw	Fresh								
	8. Kiwi fruit			3660						
	8. Leechies			3632						
	9. Watermelons			3576						
X	**9. Sweet melon**									

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
X	**10. Naartjies**	Mineola fresh		4227						
		Naartjie fresh		3558						
		Naartjie canned in syrup		3635						
X	**11. Oranges**	Fresh		3560						
	11. Grapefruit	Fresh		3546						
X	**12. Peaches**	Fresh		3565						
		Canned juice		3640						
		Canned syrup		3567						
X	**12. Nectarines**	Fresh		4228						
X	**13. Pears**	Fresh		3582						
		Canned juice		3643						
		Canned syrup		3583						
	14. Pineapple	Fresh		3581						
		Canned juice		3647						
		Canned syrup		3648						
X	**15. Plums**	Fresh		3570						
	15. Apricots			3534						
	16. Dried fruit									
	16. Dry stewed fruit									
	16. Raisins			3552						
	17. Fruit juice	Fresh								
		Sweetened								
		Unsweetened								

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	SOUPS, LEGUMES, NUTS									
X	**1. Soups**	Powdered made with water		3165						
		Vegetable		3162						
		Meat, lentil veg		3153						
		Dried bean, meat, veg		3145						
		With bones								
	2. Beans and lentils	Anything added								
	3. Nuts									
	3. Peanuts									
	Peanuts and raisins									
	FISH and SEAFOOD- BEIGE									
	1. Fried fish									
	1. Fish cakes									
	1. Fish fingers									
	1. Calamari									
Circle one	**2. Grilled/smoked/dried fish**									
	2. Haddock									
	3. Pilchards and sardines	Canned water								
	Canned	Tomato sauce								
		Mayonnaise		3488						
	3. Tuna	Brine water								

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	3. Tuna	In oil								
		Mayonnaise		3488						
	3. Pickled fish									
	MEAT- RED	*How do you eat meat? 1= with fat 2= fat trimmed*								
	1. Roast beef									
	1. Beef chops									
	1. Beef steak (bone)									
	1. Beef steak (no bone)									
	1. Beef stir-fry									
	1. Beef stew / veg			3120						
	1. Beef stew / cabbage			3006						
	2. Beef patties			2984						
	2. Mince	Regular		2987						
		With vegetables								
X	**2. Meatballs**	*Egg / no egg*								
	2. Cottage pie			3009						
	3. Burgers	Bun		3210						
	please tick, quantify, brand names (McDonalds bigmac)	Patty: Beef		2984						
		Cheese slice		2722						
		Tomato sauce		3169						
		Mayonnaise		3488						
Meal?	**Chips (fried)**	*Size?*		3740						
	Soft drink	*Size?*		3981						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	3. Hot dog	HD roll								
		Vienna								
		Tomato sauce		3169						
	3. Pita with…									
	3. Chicken burger	Bun		3210						
	please tick, quantify, name of burger (e.g KFC twister)	Crumbed, fried		3011						
		Cheese slice		2722						
		Tomato sauce		3169						
		Mayonnaise		3488						
Meal?	**Chips (fried)**	*Size?*		3740						
	Soft drink	*Size?*		3981						
	3. Chicken nuggets			3018?						
	4. Chicken stew									
	4. Fried chicken pieces	With skin								
		Without skin								
	4. Roast chicken	With skin								
		Without skin								
	4. Chicken stir-fry									
	4. Chicken other									
	6. Fat cakes and mince									
	7. Meat pies									
	7. Samoosas	Meat		3355						
		Vegetable		3414						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	7. Sausage rolls			2939						
	7. Spring rolls			2918						
	8. Mutton stew no veg									
	8. Mutton stew with veg									
	8. Mutton leg chop									
	8. Mutton loin chop			2927						
	8. Roast mutton			2947						
	9. Spare ribs			3010						
	9. Pork chops			2930						
	9. Bacon			2906						
	9. Roast pork									
	10. Pork sausages			2932						
	10. Viennas			2936						
circle	**10. Frankfurters**	*Beef/pork/chicken*								
	10. Boerewors			2931						
	11. Traditional / organ meats									
	Chicken livers			2970						
	Chicken giblets			3014						
	Chicken head			2999						
	Chicken feet			2997						
	Liver and fat			2920						
	Sheep intestine / lungs			4342						
	Shank *pork/lamb/beef*									
	Mopani worms	*How cooked?*								

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	12. Vegetarian products									
	13. Dry sausages			2949						
	13. Biltong			2912						
	VEGETABLES – GREEN									
	1. Asparagus	Fresh boiled		3695						
		Canned boiled		4094						
	2. Avocado	Fresh		3656						
	3. Baby marrows	Fresh boiled		4171						
		Fat added								
		Sauce								
	4. Beetroot	Boiled		3698						
	Grated / sliced	Salad		3699						
	Chutney, onion,	Salad								
	5. Butternut	Boiled		3759						
		Boiled Sugar added		3989						
		Sugar and fat added		4273						
	5.Pumpkin	Boiled		4164						
		Sugar and fat added		3893						
	6. Broccoli, *Fresh/frozen*	Boiled								
		Fat added		3808						
	6. Cauliflower	Boiled		3716						
		Boiled + cheddar		3715						
	Quantify white/cheese sauce Pg 23									

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	7. Cabbage	Boiled		3756						
	7. ..with potato, onion	HM		3813						
	7. ..with potato, onion	Sun oil		3815						
	7. coleslaw (mayo)			3705						
	7. Cabbage, *fried*	HM		3810						
	7. Cabbage, *fried*	Sun oil		3812						
	8. Carrots	Raw		3709						
	8. Carrots	Boiled		3757						
		Sugar added		3818						
	9. Gem squash	Boiled		3760						
		Boiled + sugar		3754						
	10. Green beans	Boiled		3696						
	10.. with potato, onion	No fat		3933						
	10.. with potato, onion	HM		3792						
	10.. with potato, onion	Sun oil		3794						
	11. Mealies	Whole, boiled		4133						
		Whole, boiled canned		4134						
	11. Sweet corn									
	12. Mixed vegetables	Canned, boiled		4264						
	carrot, corn, peas, green beans	Frozen, boiled		3727						
	caulif', carrot, green beans	Frozen, boiled		4265						
		Fresh boiled, PUM		3836						
		Fresh boiled, HM		3835						
	13. Mushrooms	Fried HM		3893						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	13. Mushrooms	Fried in Sun oil		3841						
	14. Peas	Frozen boiled		4146						
		Boiled, sugar, HM		3859						
	15. Potatoes, Baked	(ate flesh and skin)		3736						
		(ate skin only)		3970						
		(ate skin, sour cream and chives		3973						
	Boiled	(ate skin and flesh)		4155						
		(ate no skin)		3737						
		HM		3867						
		PUM		3868						
	Croquette/cake			3915						
	Fried/sautéed	HM		3871						
		Sun oil		3873						
	Frozen, boiled			4156						
	Mashed	(SM, PUM)		3875						
		(WM, HM)		3876						
	Roasted in..	.. beef fat		3878						
		.. chicken fat		3923						
		.. lamb fat		3735						
		.. pork fat		3956						
		..Sun oil		3979						
	15. Potato Salad	(mayonnaise)		3928						
	Potatoes, whole canned			4159						

Generic Sketch (look up)	A. Food items (with FPM numbers)	B. Description of food item	Tick for yes	C. Item code	D. Amount usually eaten(g) Generic/amount = g	E. Eaten every day	F. Eaten every week	G. Eaten Occasionally	H. Never eaten	
						Times/ day	Times/ week			
	16. Potato chips	Fried in Sun oil		3740						
		Oven baked		3945						
		Mayonnaise		3488						
		Tomato sauce		3169						
	If take away where from?									
	And what size?									
	17. Salad vegetables	French		3921						
		Greek		4271						
		Mixed green		3927						
	Dressing? See pg 19!									
	17. Raw tomato			3750						
	17. Cucumber			3718						
	17. Peppers raw, green			3733						
	17. Onion, raw			3755						
	17. Onion, fried	HM		3844						
	17. Onion, fried	Sun oil								
	18. Spinach/morogo	boiled		3913						
		Boiled + HM								
		Boiled + PUM								
	18.. with potato, onion	HM		3901						
	18.. with potato, onion	Sun oil								
	19. Sweet potatoes	Baked, ate skin only		3748						
		Boiled, no skin		3903						
		Sugar + HM		3749						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	20. Tomatoes, cooked	Fried, onion		3925						
		Fried, onion, peppers								
		Fried, onion, peppers, carrots								
	Okra									
	Aubergine									
	Karela									
	FATS-TAN 'prompt'									
	1. Tub margarine	Where used								
		Number of spoons								
		Number in family								
	1. Butter									
	1. Brick Margarine									
	2. White margarine (type of fat)									
	3. Cream and substitutes (brand, real dairy/plant fats)									
	4. Oils	Where used								
		Number of spoons								
		Number in family								
	5. Salad dressings	Homemade								
		Shop bought								
	5. Mayonnaise	Where used								
		Number of spoons								
		Number in family								

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
	BISCUITS, CAKE and PUDDING - PURPLE									
	1. Biscuits/cookies	Commercial plain		3216						
brand		Commercial, filling		3217						
		Homemade, plain		3341						
	2. Biscuits/savoury	Provita		3235	___ x 6					
		Ry-vita		3236	___ x 5					
		Cream crackers		3230	___ x 8					
	High fat *(circle which one)*	Tuc/salticrax/kips		3331						
	Harvest wheat	Whole wheat		3391						
	Spread?									
	Topping?									
	3. Special buns *(hot cross, Danish, raisin)*			3409						
	3. Muffins	Bran		3407						
		plain		3408						
	3. Scones	plain		3237						
	4. Tart									
	4. Cake *(circle)*	Iced/Cream								
		Plain								
	5. Doughnuts	Plain		3232						
	5. Doughnuts	Jam		3423						
	5. Doughnuts	Icing		3422						
	5. éclairs			3268						
	5. Koeksisters			3231						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	
						Times/ day	Times/ week			
X	**6. Pancakes/crumpets**	WM/Sun oil		3238						
	Toppings?									
	6. Waffles	WM/Sun oil		3238						
	Toppings?									
	7. Baked pudding									
	7. Trifle									
	7. Custard									
	7. Instant pudding									
	8. Rusks	Commercial		3329						
	9. Special breads	Banana loaf		3333						
		Date loaf		3256						
		Raisin bread		3214						
	Spread?									
	SNACKS, SWEETS and COLD DRINKS-PINK									
	1. Carbonated cold drinks e.g. coke			3981						
	1. Diet cold drinks e.g. diet coke			3990						
	2. Mageu			4056						
	2. Cold drinks (powder)									
	2. Energy drinks			4007						
	2. Squashes			3982						

Generic Sketch (look up)	**A.** Food items (with FPM numbers)	**B**. Description of food item	**Tick for yes**	**C**. Item code	**D**. Amount usually eaten(g) Generic/amount = g	**E**. Eaten every day	**F**. Eaten every week	**G**. Eaten Occasionally	**H.** Never eaten	**Generic Sketch** (look up)
						Times/ day	Times/ week			
brands	**3. Crisps, potato** *(lays)*			3417						
Size packets	**3. Crisps Savoury,** *(nik naks, fritos, ghost pops)*			3418						
	3. Popcorn, plain			3332						
	3. Popcorn, sugar coated			3359						
	4. Sweets									
	Lollipops									
Brands	**4. Chocolates**									
describe										
sizes										
	SAUCES and CONDIMENTS-GREY									
	1. Cheese sauce	LFM, HM, cheese		3126						
		LFM, PUM, cheese		3127						
		SM, PUM, cheese		3128						
		WM, HM, cheese		3125						

Generic Sketch (look up)	A. Food items (with FPM numbers)	B. Description of food item	Tick for yes	C. Item code	D. Amount usually eaten(g) Generic/amount = g	E. Eaten every day	F. Eaten every week	G. Eaten Occasionally	H. Never eaten	
						Times/ day	Times/ week			
	1. White sauce	LFM, HM		3143						
		LFM, PUM		3164						
		SM, PUM		3141						
		WM, HM		3142						
	2. Chakalaka									
	2. Atjar			3117						
	2. Tomato sauce and other									
	2. Chutney									
	ALCOHOLIC DRINKS-GREY									
	1. Beer (regular/low alcohol)									
	1. Cider									
	Coolers									
	2. Wine									
	2. Champagne									
	3. Spirits (any carbonated drink e.g. coke added)									
	4. Liqueurs and Fortified wine									
	OTHER									

APPENDIX 3

- **UV EXPOSURE AND GENERAL HEALTH QUESTIONNAIRE**

Study of Vitamin D and Cardiometabolic risk

UV exposure and general health questionaire

DATE: **Day** ☐☐ **Month** ☐☐ **Year** ☐☐☐☐

☐☐☐☐☐☐☐

☐☐☐

DCR Id:_____ **Barcode**

DATE: ____/_____/2011

SECTION ONE: PERSONAL DETAILS

Firstly we would like to ask you some questions about your background:

1. Gender: **Male** **Female**
2. What is your age **Age** in **years** ----------
3. In what country were you born? _____
4. **IF YOU WERE NOT BORN IN SOUTH AFRICA**
   In what year did you come to live in South Africa? _____

5. We are interested in your parents' **ETHNIC ORIGIN** (that is, the place where most of their ancestors come from) and the **COUNTRY THEY WERE BORN IN.**

	Country of Birth	**Ethnic Origin (Please tick the appropriate box)**
MOTHER		□Asian □African
		□Other □Don't know
FATHER		□Asian □African
		□Other □Don't know

6. What is the **highest** technical, professional or academic **qualification** you have **completed?**

(Please circle the appropriate school)

Did not complete primary school

Primary school

Did not complete high school

Matric

Technicon

Bachelors degree

Post graduate

7. Please circle which best describes your main occupation:

Mainly indoors

Half indoors and half out doors

Mainly outdoors

SECTION TWO : Smoking. Other risk factors

8. Do you, or have you ever regularly smoked cigarettes, cigars or a pipe (more than one a day for more than six months)?

☐No. I have never smoked regularly (Please go to Question 9).

☐Yes. I am a current smoker.

☐Yes. I am an ex-smoker.

IF YES…

9(a). On average, how many cigarettes, cigars or pipes do you smoke or did you used to smoke each day? ☐

9(b). At what age did you start smoking? ☐ years

9(c). At what age did you stop smoking? ☐years

10. Overall how would you rate your **GENERAL HEALTH** over the **PAST MONTH**?

Excellent Very good Good Fair Poor Very poor

11. Have you ever had surgery to remove all or part of the Thyroid gland in your neck? ☐Yes ☐No

12. Have you ever had surgery on your neck? ☐Yes ☐No
If YES, please explain:_____

13. Have you ever had radiation therapy on your neck? ☐Yes ☐No
(Not an X-Ray)
If YES, please explain:_____

14. Have you suffered from a heart attack before? ☐Yes ☐No
If yes how were you treated for it?
Surgery
Medication
Both

15. Have any of you immediate family suffered from a heart attack. ☐Yes ☐No
If yes how are you related?
Mother Father Brother Sister
At what age did this happen

Date: _____**/**_____**/**_____

Ask each volunteer the following questions:

	Time Outdoors			Amount of Skin Exposed			
	<5 min	5-30 min	>30min	Hands and face	Hands, face, arms	Hands, face, legs	Bathing suit
Monday	0	1	2	1	2	3	4
Tuesday	0	1	2	1	2	3	4
Wednesday	0	1	2	1	2	3	4
Thursday	0	1	2	1	2	3	4
Friday	0	1	2	1	2	3	4
Saturday	0	1	2	1	2	3	4
Sunday	0	1	2	1	2	3	4

Circle time outdoors and skin exposed for each day in last week

---

Day	**Body area**	**Clothing**	**Circle which item is worn**	**Sunscreen used? If Yes add √ below**	**Skin lightening agent used? If Yes add √ below**
Monday	Head	Nothing	4		
		Beanie/bike helmet	3		
		Baseball cap	2		
		Cowboy hat	1		
	Torso/arms	Nothing	47		
		Bikini top/sports bra	42		
		Tank top	18		
		Tee shirt	10		
		Quarter length shirt	3		
		Long sleeves	0		
	Legs	Bikini bottom	38		
		Short shorts/ Skirt	24		
		Knee-length shorts/skirt	8		
		Full-length pants	0		
	Feet	Nothing	2		
		Sandals	1.5		
	_____	Shoes _____	0 _____		
Tuesday	Head	Nothing	4		
		Beanie/bike helmet	3		
		Baseball cap	2		
		Cowboy hat	1		

	Torso/arms	Nothing	47		
		Bikini top/sports bra	42		
		Tank top	18		
		Tee shirt	10		
		Quarter length shirt	3		
		Long sleeves	0		
	Legs	Bikini bottom	38		
		Short shorts/ Skirt	24		
		Knee-length shorts/skirt	8		
		Full-length pants	0		
	Feet	Nothing	2		
		Sandals	1.5		
		Shoes	0		
Wednesday	Head	Nothing	4		
		Beanie/bike helmet	3		
		Baseball cap	2		
		Cowboy hat	1		
	Torso/arms	Nothing	47		
		Bikini top/sports bra	42		
		Tank top	18		
		Tee shirt	10		
		Quarter length shirt	3		
		Long sleeves	0		
	Legs	Bikini bottom	38		
		Short shorts/ Skirt	24		
		Knee-length shorts/skirt	8		
		Full-length pants	0		
	Feet	Nothing	2		
		Sandals	1.5		
		Shoes	0		
Thursday	Head	Nothing	4		
		Beanie/bike helmet	3		
		Baseball cap	2		
		Cowboy hat	1		
	Torso/arms	Nothing	47		
		Bikini top/sports bra	42		
		Tank top	18		
		Tee shirt	10		

		Quarter length shirt	3		
		Long sleeves	0		
	Legs	Bikini bottom	38		
		Short shorts/ Skirt	24		
		Knee-length shorts/skirt	8		
		Full-length pants	0		
	Feet	Nothing	2		
		Sandals	1.5		
		Shoes	0		
Friday	Head	Nothing	4		
		Beanie/bike helmet	3		
		Baseball cap	2		
		Cowboy hat	1		
	Torso/arms	Nothing	47		
		Bikini top/sports bra	42		
		Tank top	18		
		Tee shirt	10		
		Quarter length shirt	3		
		Long sleeves	0		
	Legs	Bikini bottom	38		
		Short shorts/ Skirt	24		
		Knee-length shorts/skirt	8		
		Full-length pants	0		
	Feet	Nothing	2		
		Sandals	1.5		
		Shoes	0		
Saturday	Head	Nothing	4		
		Beanie/bike helmet	3		
		Baseball cap	2		
		Cowboy hat	1		
	Torso/arms	Nothing	47		
		Bikini top/sports bra	42		
		Tank top	18		
		Tee shirt	10		
		Quarter length shirt	3		
		Long sleeves	0		
	Legs	Bikini bottom	38		
		Short shorts/ Skirt	24		

		Knee-length shorts/skirt	8		
		Full-length pants	0		
	Feet	Nothing	2		
		Sandals	1.5		
		Shoes	0		
Sunday	Head	Nothing	4		
		Beanie/bike helmet	3		
		Baseball cap	2		
		Cowboy hat	1		
	Torso/arms	Nothing	47		
		Bikini top/sports bra	42		
		Tank top	18		
		Tee shirt	10		
		Quarter length shirt	3		
		Long sleeves	0		
	Legs	Bikini bottom	38		
		Short shorts/ Skirt	24		
		Knee-length shorts/skirt	8		
		Full-length pants	0		
	Feet	Nothing	2		
		Sandals	1.5		
		Shoes	0		

Circle which item of clothing worn and use of sunscreen/skin lightener for each day in last week.

Additional information:

Completed by----------

APPENDIX 4

- **PATIENT INFORMATION/INFORMED CONSENT**

PATIENT INFORMATION

INTRODUCTION

My name is Jaya George and I am a doctor working in the Department of Chemical pathology. You are invited to consider participating in a research study. Your participation in this study is entirely voluntary.

Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, the study procedures, benefits, risks, and precautions as well your right to withdraw from the study at any time.

This information leaflet is to help you to decide if you would like to participate. You should fully understand what is involved before you agree to take part in this study.

If you have any questions, do not hesitate to ask me.

PURPOSE OF THE STUDY

The project is aimed at studying the vitamin D levels in various ethnic groups in this province and to compare these levels will internationally recommended levels. Vitamin D is important for normal bone strength as well as for normal immune function.

We will also measure the levels of calcium, magnesium, parathyroid hormone, creatinine and alkaline phosphate. Changes in vitamin D levels will affect the levels of these in blood. Some blood will also be stored to check for DNA for changes in the vitamin D receptor.

LENGTH OF STUDY AND NUMBER OF PARTICIPANTS

Approximately 600 participants will be recruited to participate in the study.

The total amount of time required for your participation will be a maximum of 4 hours and you will not be expected return for follow up.

PROCEDURES

If you agree to take part in this study, you will first be asked questions and examined to see if you qualify for this study.

A few tubes of blood will be collected from a vein in your arm. This is equal to about two tablespoons (30 mls) of blood. The samples will be given a unique number to protect your privacy.

Thereafter, you will have your weight, height, waist circumference and blood pressure measured and recorded. You will also have a scan to assess the density of your bones and an ultrasound scan for fat.This information will be used to assess if body mass index has any impact on vitamin D levels.

WILL ANY OF THESE STUDY PROCEDURES RASULT IN DISCOMFORT OR INCONVENIENCE?

Drawing of blood may cause some discomfort associated with pain, bruising or bleeding at the puncture site. The procedure will be performed under sterile conditions to ensure your protection.

BENEFITS
Your participation in this study will assist in assessing the status of vitamin D in the different population groups in this province.

RIGHTS AS A PARTICIPANT IN THIS STUDY

Voluntarily
Your participation in the study is entirely voluntary and you may decline to participate.

Withdrawal
You may withdraw at any time and your withdrawal will not affect access to other medical care.

FINANCIAL ARRANGMENTS
You will not be required to pay for any of the study related procedures.

REIMBURSMENT FOR STUDY PARTICIPATION

You will not be paid to participate in the study.

ETHICAL APPROVAL
This study has been approved by the Human Ethics Committee of the University of the Witwatersrand, the clearance number

SOURCES OF ADDITIONAL INFORMATION
If at any time after you feel that you have questions regarding the study, please do not hesitate to the principal investigator , Dr Jaya George, at this number: 0828820132.

CONFIDENTIALITY

All information obtained during the course of the study including hospital records, personal data and research data will be kept strictly confidential. Data that may be reported in scientific or medical journals will not include any information that identifies you as a participant in this study.

Any information uncovered regarding your test results or state of health as a result of your participation in the study will only be made known to your treating doctors. You will be informed of any findings of importance to your health during participation in this study.

INFORMED CONSENT DCR ID

I hereby confirm that the researcher, has explained the nature, conduct, benefits and risks of this research project.

- I have also received, read and understood the above written information regarding the study.

- I am aware that the results of the study, including personal details regarding my age, sex and diagnosis will anonymously be processed into a study report.

- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher appearing on the first page of this document.

- I may at any stage, without prejudice, withdraw my consent and participation in the study.

- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study

PARTICIPANT

Printed Name	**Signature / Name or Thumbprint**	**Date**

WITNESS (if applicable)

Printed Name	**Signature / Name or Thumbprint**	**Date**

Researcher Name **Signature**

Date

APPENDIX 5

- **ANTHROPOMETRY/MEASUREMENTS**

University of the Witwatersrand

ANTHROPOMETRY/MEASUREMENTS

DATE : Day ☐☐ **Month** ☐☐ **Year** ☐☐☐☐

Patient number : ☐☐☐■☐

Medications:

Have you taken any medication for the following conditions in the past 6 months?

Diabetes	Yes	No
High blood pressure	Yes	No
Cholesterol	Yes	No

Have you been on any other medication for the past 6 months?

If yes: for what conditions(please list)

MEASUREMENTS

ANTHROPOMETRY

- STANDING HEIGHT: (mm) ☐☐☐☐
- WEIGHT: (kg) ☐☐☐•☐
- WAIST CIRCUMFERENCE: (mm) ☐☐☐☐
- HIP CIRCUMFERENCE: (mm) ☐☐☐☐
- TANITA measured ☐Y ☐N

  (Attach to file)

BLOOD PRESSURE

- SYSTOLIC BP
- DIASTOLIC BP

☐☐☐ ☐☐☐ ☐☐☐

☐☐☐ ☐☐☐ ☐☐☐

☐☐☐ ☐☐☐ ☐☐☐

- PULSE

		h		

Research Assistant name: | | Date: | |

Notes:

APPENDIX 6

- **PUBLICATION**

  The association of 25 hydroxyvitamin D and parathyroid hormone with metabolic syndrome in two ethnic groups in South Africa

OPEN ACCESS | Freely available online

PLOS ONE

The Association of 25 Hydroxyvitamin D and Parathyroid Hormone with Metabolic Syndrome in Two Ethnic Groups in South Africa

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Abstract

Introduction: Though inconsistent, a number of studies have shown an association between vitamin D (25(OH)D) status, parathyroid hormone (PTH) and the metabolic syndrome (Met S). These have largely been carried out in Caucasians or black subjects living in high income countries. There no data on the relationship of 25(OH)D and PTH status with Met S in populations resident in Africa. The aims of this study were to evaluate if there was an association of 25(OH)D or PTH with Met S in non-Caucasian populations in South Africa, and whether these molecules explained ethnic differences in the prevalence of Met S and its individual components.

Methods: We measured anthropometry, serum 25(OH)D and PTH levels and the components of Met S, plus related metabolic variables, in 374 African and 350 Asian Indian healthy adults from the greater Johannesburg metropolitan area.

Results: Met S was diagnosed in 29% of the African and 46% of the Asian Indian subjects ($p<0.0001$). Subjects with Met S had higher PTH than those without Met S, ($p<0.0001$), whilst 25(OH)D levels were not significantly different ($p=0.50$). In multivariate analysis, 25(OH)D was not associated with any components of the Met S however PTH was shown to be positively associated with systolic ($p=0.018$) and diastolic ($p=0.005$) blood pressures and waist circumference ($p<0.0001$) and negatively associated with HOMA ($p=0.0008$) levels. Logistic regression analysis showed that Asian Indian ethnicity (OR 2.24; 95% CIs 1.57, 3.18; $p<0.0001$) and raised PTH (OR 2.48; 95% CIs 1.01, 6.08; $p=0.04$; adjusted for 25(OH)D) produced an increased risk of Met S but 25(OH)D did not (OR 1.25; 95% CI 0.67, 2.24; $p=0.48$).

Conclusions: Plasma PTH but not 25(OH)D is an independent predictor of the Met S in African and Asian Indians in South Africa.

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Competing Interests: Siemens Diagnostics provided PTH and insulin kits to this study. HEvD is employed by Lancet Laboratories, South Africa (a private pathology laboratory). This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

The incidence of obesity is on the rise in many low and middle income countries (LMICs) [1] and South Africa is no exception, with a national survey showing that 60% of South African women were either overweight or obese [2]. Obesity is associated with an increased risk of cardiovascular disease (CVD) and additional risk factors include hyperglycaemia or type 2 diabetes, hypertriglyceridemia, low high-density lipoprotein cholesterol (HDL-C), and hypertension. These CVD risk factors are commonly found together in subjects with abdominal obesity and this grouping together of CVD risk factors has been termed the metabolic syndrome (Met S) [3].

The burden of disease related to CVD is expected to increase substantially in LMIC, such as South Africa, over the next decades and it is important to understand traditional and non-traditional risk factors that contribute to this disease burden. Some populations are more susceptible to CVD because of specific factors – e.g. familial hypercholesterolaemia in the South African Afrikaner population [4] or insulin resistance in the Indian population [5]. The prevalence of cardiovascular and diabetes risk factors is higher in Asian Indian than African population groups in South Africa despite greater obesity in the latter population [6].

It has been suggested from several studies in various parts of the world that vitamin D deficiency or insufficiency may increase risk of the Met S and its sequelae, type 2 diabetes and CVD [7,8]. Vitamin D is a hormone precursor which before exerting its metabolic effects undergoes two successive hydroxylation steps. The first converts vitamin D to 25 hydroxyvitamin D (25(OH)D) and the second converts 25(OH)D to the main active hormonal form 1,25 dihydroxy vitamin D (1,25-$(OH)_2$D). Hypovitaminosis D appears to exert its effects via reductions in intracellular calcium

and through the effects of 1,25(OH)$_2$D on the regulation of various target genes e.g. by lack of suppression of the renin gene leading to hypertension [9], and reduced insulin secretion through reduction in islet β –cell function [8].

Elevated parathyroid hormone (PTH) levels have also emerged as a potential risk factor for Met S [10,11]. PTH has been shown to be inversely associated with insulin sensitivity [12] and directly with blood pressure [13,14]. Both 25(OH)D and PTH are calciotrophic hormones and their metabolism is closely related such that a decrease in serum 25(OH)D leads to a rise in PTH [15]. Currently, it is unclear whether the association noted between hypovitaminosis D and components of the Met S is mediated by 25(OH)D or PTH or by both.

There are no studies from Africa that have investigated any associations between 25(OH)D, PTH and components of the Met S. Therefore, the aims of this study were to examine the association of 25(OH)D and PTH with Met S in African and Asian Indian population groups in South Africa and to determine whether ethnic differences in 25 (OH)D or PTH levels contribute to differences in the prevalence of Met S, its component parts and related metabolic variables, within these populations. The hypotheses to be tested were that 25(OH)D is lower and PTH is higher in Met S patients in both population groups and that ethnic differences in the prevalence of Met S and its component parts are related to ethnic differences in the blood level of 25(OH)D.

Research Design and Methods

Subjects

This was a cross sectional study of African and Asian Indians from the greater Johannesburg-Soweto metropolitan area in South Africa. Participants were recruited via the Birth to Twenty study, which is a longitudinal analysis of more than 3200 children and their caregivers which began in 1990 and is representative of long-standing urban inhabitants [16]. Caregivers of the cohort were contacted and asked if they had any family members or neighbours that would be interested in participating. Exclusion criteria consisted of pregnancy, breast feeding, renal failure, age below 18 or above 70, Caucasian or persons of mixed ancestry. A total of 730 participants were recruited, and were stratified by ethnicity, age and sex. Ethnicity was self-reported as African or Asian Indian. Ethics clearance was obtained from the Human Ethics Committee of the University of the Witwatersrand and written informed consent was obtained from each subject.

Biochemistry

After an overnight fast of at least 8 hours, peripheral venous blood was collected in EDTA tubes for 25(OH)D, PTH and glycated haemoglobin analysis (HbA1c), in fluoride tubes for glucose and in plain tubes for the rest of the analytes. Tubes were kept on ice and centrifuged after 30 minutes at 3500 g for 10 minutes. Glucose, HbA1c and lipid profile were assayed on the day of collection and aliquots for 25(OH)D, PTH, and insulin were stored at −80°C until analysis. Glucose, total cholesterol, HDL-C and triglycerides were measured enzymatically on an automated analyser (Siemens ADVIA 1800, Tarrytown, USA). The HbA1c was measured on the ADVIA 1800, which was standardized according to the Diabetes Control and Complications Trial National Glycohemoglobin Standardization Program [17]. Creatinine was measured by a modified Jaffe test and fasting insulin and PTH were measured in batches by a chemiluminescence assay on the ADVIA Centaur (Siemens Diagnostics, Tarrytown, USA). The intra assay intra assay coefficient of variation (CV) for PTH ranges from 5.2% at 4.3 pmol/L to 3.4% at 23.7 pmol/L.

Plasma 25(OH)D was measured using the Clin Rep high performance liquid chromatography (HPLC) kit (Recipe, Munchen, Germany). In this analytical method, 25(OH)D was determined from plasma using HPLC with a photodiode array (PDA) detector. Prior to HPLC analysis a short liquid-liquid extraction was performed. Thereafter the samples were injected onto the HPLC system and the analytes were separated on the appropriate analytical column. The separated 25(OH)D$_2$ and 25 (OH)D$_3$ were detected at a wavelength of 264 nm. The chromatograms were integrated using peak height. Total vitamin D (25(OH)D) was taken as the sum of 25(OH)D$_2$ and 25(OH)D$_3$. The intra assay CV for 25(OH)D ranged from 0.9 4.9% and the inter-assay CV ranged from 3.0- 4.9%.The limit of detection was 2.5 nmol/l for 25(OH)D$_3$ and 7.5 nmol/L for 25(OH)D$_2$. Our laboratory participates in the vitamin D external quality assurance scheme (DEQAS).

Diagnosis of Metabolic Syndrome, and Measurement of Insulin Resistance and Glomerular Filtration Rate

Met S was determined using the harmonized definition [3,17]. Thus, subjects had to have at least three of the following criteria: elevated waist circumference (≥94 cm for African males, ≥80 cm for African or Asian Indian females, ≥90 cm for Asian Indian males); elevated serum triglycerides (≥1.7 mmol/L); reduced serum HDL-C (<1.0 mmol/L for males and 1.3 mmol/L for females); elevated blood pressure (systolic blood pressure ≥130 mmHg and/or diastolic pressure ≥85 mmHg) and elevated fasting blood glucose (≥5.6 mmol/L). Insulin resistance was measured using the homeostasis model assessment (HOMA) technique [18] and glomerular filtration rate (GFR) was calculated by a modified MDRD formula [19].

Medical History and Anthropometric Data Collection

A standardized questionnaire was used to collect information on age, sex, visit date, smoking (current, former, never), self-reported diabetes, hypertension, dyslipidaemia, coronary heart disease and medication use. Body weight and height were measured with subjects in light clothing and without shoes using an electronic scale and wall mounted stadiometer (Holtain, UK). The waist circumference was measured with a soft measuring tape to the nearest 0.5 cm at the level of the smallest girth above the umbilicus in the standing position. Blood pressure was measured thrice in the right arm, with the subject seated and after 5 minutes rest using an automated sphygmomanometer. The final reading was used.

Statistical Methods

Stata 12 (College Station, TX, USA) was used for all analyses. The distribution of variables was assessed by the Shapiro-Wilk's W test and by examination of the normal probability plot. Logarithmic transformations were applied for all non-normally distributed variables. In descriptive analysis, normally distributed, continuous variables were reported as means ± SD whilst non-normally distributed data were expressed as median (interquartile range). Categorical data were reported as N (percent).

Pearson's correlation was used to assess the association of 25(OH)D and PTH with the selected variables. Multivariate regression analyses were conducted to assess the association of the Met S components with 25(OH)D and PTH after adjusting for potential confounders i.e. age, gender and body mass index (BMI). Ethnicity was also included as an independent variable to

Table 1. Characteristics of subjects according to ethnicity and gender.

Variable	African	Asian Indian	p-values	African Males	African Females	p-values	Asian Indian Males	Asian Indian Females	p-values
N	373	344	–	181	192	–	161	183	–
Age (years)	41.6±13.1	43.5±12.9	0.06	42.0±13.2	42.0±13.1	0.9	42.0±13.2	46.0±12.7	0.5
Glucose (mmol/L)	4.9 (4.6, 5.0)	5.21 (4.80, 5.70)	<0.0001	4.90 (4.60, 5.40)	4.90 (4.60, 5.30)	0.5	5.20 (4.91, 5.80)	5.10 (4.80, 5.63)	0.03
Triglycerides (mmol/L)	0.85 (0.62, 1.27)	1.26 (0.83, 1.85)	<0.0001	0.93 (0.67, 1.42)	0.82 (0.57, 1.17)	0.03	1.37 (1.00, 2.08)	1.10 (0.77, 1.60)	<0.0001
HDL-C (mmol/L)	1.28 (1.08, 1.53)	1.26 (1.07, 1.53)	0.81	1.20 (1.01, 1.43)	1.33 (1.17, 1.59)	<0.0001	1.12 (1.00, 1.26)	1.45 (1.24, 1.73)	<0.0001
Systolic blood pressure (mmHg)	131 (120, 146)	123 (113, 134)	<0.0001	133 (121, 148)	129 (119, 143)	0.16	125 (118, 135)	118 (108, 132)	0.0003
Diastolic blood pressure (mmHg)	83 (76, 93)	80.0 (73.1, 87.0)	<0.0001	82.0 (74.0, 93.0)	83.0 (77.3, 93.2)	0.40	82.0 (75.3, 89.1)	78.2 (72.0, 85.2)	0.006
Waist (cm)	89.0 (78.0, 102)	95.0 (85.0, 106)	0.0001	85.0 (75.0, 99.0)	95.0 (82.0, 105)	<0.0001	98.0 (90.5, 108)	93.0 (82.0, 103)	0.0006
GFR (ml/min/1.73 m²)	88.0±22.4	88.1±19.7	0.9	88.7±28.7	87.4±22.5	0.92	84.2±17.4	92.9±20.4	<0.0001
Calcium (mmol/L)	2.27±0.09	2.26±0.11	0.21	2.28±0.08	2.27±0.10	0.32	2.26±0.1	2.26±0.1	0.63
Cholesterol (mmol/L)	4.22±1.04	4.97±1.10	<0.0001	3.93±0.99	4.24 1.07	0.002	4.72±1.17	4.97±1.04	0.16
BMI (kg/m²)	26.2 (21.7, 31.7)	26.7 (23.3, 31.0)	0.38	23.3 (20.2, 27.3)	29.9 (24.3, 35.3)	<0.0001	26.2 (23.7, 30.4)	27.1 (23.0, 32.8)	0.36
HOMA	1.83 (1.1, 2.9)	3.23 (2.13, 5.01)	<0.0001	1.43 (0.80, 2.69)	2.16 (1.31, 2.92)	0.0001	3.45 (2.15, 5.50)	3.15 (2.05, 4.60)	0.12
PTH (pmol/L)	4.70 (3.3, 6.5)	4.85 (3.61, 6.92)	0.09	4.30 (3.00, 5.60)	5.30 (3.80, 5.90)	0.0002	4.60 (3.60, 6.40)	5.00 (3.70, 7.20)	0.09
25 (OH) D (nmol/L)	70.9 (51.5, 95.1)	41.8 (28.6, 56.8)	<0.0001	72.7 (51.1, 94.1)	58.3 (42.9, 85.6)	0.0008	46.8 (33.6, 62.7)	35.7 (23.0, 54.5)	0.0002

Data given as mean ± SD for normally distributed data and median (IQR) for non-normally distributed data.

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determine if ethnic differences in Met S components were mediated by 25(OH)D or PTH.

In order to determine if the higher risk of Met S in the Indian population was mediated by 25(OH)D or PTH, logistic regression analysis was performed with the stepwise addition of 25(OH)D and PTH to a model that also included ethnicity, age, gender and BMI as independent variables. The attenuation of the p-value (from significant to non-significant) for the ethnicity odds ratio was used as the indicator of any effect.

PTH was found to increase the risk of Met S and therefore we analysed which of the individual components of the Met S were influenced by PTH by separately adding each individual component to a logistic regression model for MetS risk which included PTH, age, gender, ethnicity, BMI and 25(OH)D as independent variables. As described above the attenuation of the p-value, in this case for the PTH odds ratio, was used as the effect indicator.

In all regression models Africans were coded as 0 and Asian Indians as 1whilst females were coded as 0 and males as 1.

Results

Six participants were excluded from the analyses due to incomplete data collection and seven participants with serum calcium above the normal reference range (2.15–2.50 mmol/L) were also excluded. Characteristics of the participants (n = 717) are shown in Table 1. Both ethnic groups had similar ($p>0.05$) age, BMI, HDL-C, GFR, calcium and PTH levels. Asian Indian subjects had significantly higher fasting glucose, triglycerides and total cholesterol ($p<0.0001$ for each of the 3 comparisons) compared to the African population. They also had a greater waist circumference ($p = 0.0001$) and were more insulin resistant ($p<0.0001$). African subjects had higher 25(OH)D levels and systolic and diastolic blood pressures ($p<0.0001$ for all three comparisons). The prevalence of 25(OH)D deficiency (defined as 25(OH)D <30 nmol/L [20]) was 3.0% in the African and 15.0% in the Asian Indian population ($p<0.0001$). The month of the year during which blood samples were drawn for 25(OH)D measurement may be a confounding variable since 25(OH)D levels are known to vary across the seasons [21]. However, ethnic differences in plasma 25(OH)D levels were still observed irrespective of the season in which the blood sample was obtained. Vitamin D levels were highest for both ethnic groups during the autumn months (data not shown).

In both ethnic groups females had lower 25(OH)D levels compared to their male counterparts ($p = 0.0008$ in Africans and $p = 0.0002$ in Asian Indians) and this was also observed for triglyceride levels ($p = 0.03$ in Africans and $p<0.0001$ in Asian Indians) (Table 1). The PTH levels were higher in African females than males ($p = 0.0002$), and although the same pattern was observed in Asian Indians, the gender effect was not significant ($p = 0.09$). No gender differences were observed for age or calcium levels in either ethnic group. In African females, cholesterol ($p = 0.002$), BMI ($p<0.0001$) and HOMA ($p = 0.0001$) were higher than in males, but no gender effect for these variables was seen in the Indian population. Asian Indian females had higher GFR than Asian Indian males ($p<0.0001$) but no gender difference was observed in the African cohort. In Asian Indian males, glucose

Table 2. Prevalence of individual components of the Met S in each ethnic group.

Variable	Africans with Met S (N = 109)	Asian Indians with Met S (N = 161)	p-values
Raised glucose	48 (44.0)	97 (60.3)	0.009
Raised triglycerides	40 (36.7)	88 (54.7)	0.004
Reduced HDL-C	74 (67.9)	91 (61.5)	0.28
Raised systolic blood pressure	96 (88.9)	121 (75.2)	0.005
Raised diastolic blood pressure	83 (76.9)	110 (68.3)	0.13
Raised waist circumference	103 (94.5)	156 (96.9)	0.33

Data given as N (%).
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(p = 0.03) and systolic (p = 0.0003) and diastolic (p = 0.006) blood pressure were higher than in females but no differences were noted between sexes in the African subjects. Within both ethnic groups, HDL-C levels were higher in females than males (p<0.0001 for both). Waist circumference was significantly higher in African females than males (p<0.0001), but in the Asian Indian subjects this trend was reversed (p = 0.0006).

Met S was diagnosed in 38% of the participants, with a prevalence of 29% in the African and 46% in the Asian Indian group (p<0.0001). The data in Table 2 show that within each ethnic group the most prevalent Met S component was elevated waist circumference (94.5% in Africans and 96.9% in Asian Indians; p = 0.33) and elevated triglyceride was the least common (36.7% in Africans and 54.7% in Asian Indians: p = 0.004). The occurrence of raised fasting glucose levels was more common in Asian Indian than African Met S patients (p = 0.009), whilst raised systolic blood pressure was more common in African than Asian Indian Met S subjects (p = 0.005). Low HDL-C (p = 0.28) and raised diastolic blood pressure (p = 0.13) were not significantly different in frequency across the two ethnic groups.

In both ethnic groups, subjects with Met S had higher ages, HbA1c, HOMA, PTH, cholesterol and BMI measures than those without the Met S, whilst GFR was lower in those with Met S (p<0.05 for all comparisons) (see Table 3). Calcium (p = 0.80) and 25(OH)D levels (p = 0.50) were not significantly different across these 2 groups.

In a univariate analysis there was a significant negative correlation between PTH and 25(OH) D (p<0.0001, see Table 4) and a significant trend for PTH to be higher in Indian

Table 4. Pearson's correlations for PTH and 25(OH)D for all subjects.

Variable	Log PTH	Log 25(OH)D
Age	0.26 (<0.0001)	−0.02(0.52)
Log BMI	0.22 (0.0001)	0.03 (0.38)
GFR	−0.07 (0.05)	−0.11 (0.003)
Log Glucose	0.09 (0.02)	−0.06 (0.09)
Cholesterol	0.11 (0.005)	−0.17 (<0.0001)
Log Triglyceride	0.14 (<0.0001)	−0.08 (0.02)
HDL-C	0.009 (0.81)	−0.06 (0.08)
Log Systolic blood pressure	0.15 (0.0001)	0.12 (0.001)
Log Diastolic blood pressure	0.19 (0.0001)	0.08 (0.02)
Log HOMA	0.09 (0.02)	0.02 (0.67)
Log PTH	-	−0.19 (<0.0001)
Log 25(OH)D	0.24 (<0.0001)	-
Log Waist	0.25 (<0.0001)	0.0002 (0.99)

Data given as r (p-value).
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Table 3. Comparison of subjects with and without Met S within each ethnic group.

	Africans without Met S	Africans with Met S	p-values	Asian Indians without Met S	Asian Indians with Met S	p-values
N	260	109	-	186	158	-
Age (years)	39.1±12.9	47.7±11.3	<0.0001	38.4±12.7	49.4±10.5	<0.0001
HbA1c (%)	5.31 (5.06, 5.32)	5.64 (5.35, 6.36)	<0.0001	5.33 (5.04, 5.56)	5.76 (5.41, 6.13)	<0.0001
HOMA	1.44 (0.87, 2.35)	2.85 (2.00, 4.00)	<0.0001	2.43 (1.72, 3.58)	4.56 (2.99, 6.77)	<0.0001
PTH (pmol/L)	4.50 (3.25, 5.95)	5.41 (3.92, 7.60)	0.0002	4.40 (3.50, 6.20)	5.40 (4.13, 7.62)	0.0001
25(OH)D (nmol/L)	72.3 (52.6, 95.4)	67.4 (49.9, 94.8)	0.45	48.3 (34.8, 66.0)	50.5 (34.7, 70.1)	0.50
GFR ml/min/1.73 m²	91.3±23.0	84.2±19.3	<0.0001	93.1±20.2	87.2±19.1	0.003
Calcium (mmol/L)	2.27±0.09	2.28±0.09	0.30	2.26±1.11	2.27±0.10	0.80
Cholesterol (mmol/L)	4.02±0.95	4.48±1.05	0.001	4.86±1.03	5.11±1.17	0.04
BMI (kg/m²)	24.0 (20.0, 29.0)	30.9 (27.4, 35.1)	<0.0001	24.9 (21.1, 29.6)	28.8 (25.3, 32.6)	<0.0001

Data given as mean ±SD for normally distributed data and median (IQR) for non-normally distributed data.
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Table 5. Multiple regression models for determining the effects of ethnicity, PTH and 25(OH)D on components of the metabolic syndrome.

Dependent variable	Model numbers and independent variables					
	MODEL 1	MODEL 2		MODEL 3		
	Ethnicity	Ethnicity	25(OH)D (log)	Ethnicity	25(OH)D (log)	PTH (log)
Log Glucose	0.02 (0.013)	0.02 (0.015)	−0.003 (0.47)	0.02 (0.015)	−0.003 (0.44)	−0.01 (0.43)
Log Triglycerides	0.13 (<0.0001)	0.13 (<0.0001)	−0.001 (0.89)	0.13 (<0.0001)	0.0007 (0.94)	0.04 (0.32)
Log HDL-C	0.0002 (0.99)	−0.0007 (0.93)	−0.007 (0.11)	−0.0009 (0.93)	−0.006 (0.175)	−0.005 (0.78)
Log Systolic blood pressure	−0.04 (<0.0001)	−0.04 (<0.0001)	0.002 (0.34)	−0.04 (<0.0001)	0.002 (0.28)	0.02 (0.018)
Log Diastolic blood pressure	−0.02 (<0.0001)	−0.02 (<0.0001)	0.003 (0.14)	−0.02 (<0.0001)	0.004 (0.11)	0.03 (0.005)
Log Waist	0.02 (0.002)	0.02 (0.001)	0.003 (0.37)	0.02 (0.005)	0.004 (0.19)	0.09 (<0.0001)
Log HOMA	0.23 (<0.0001)	0.23 (<0.0001)	0.0009 (0.93)	0.23 (<0.0001)	−0.0003 (0.97)	−0.13 (0.008)

Data given as beta (p-value); all models adjusted for age, gender and BMI. Ethnicity, 25(OH) D and PTH were added in a forward, stepwise manner.
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subjects ($p = 0.04$) and females ($p = 0.0002$). We also observed significant ($p<0.05$) positive associations of PTH with age, BMI, glucose, HOMA, triglyceride, cholesterol, waist circumference and systolic and diastolic blood pressures. The 25(OH)D levels were correlated negatively with BMI, and GFR and correlated positively with systolic and diastolic blood pressure ($p<0.05$ for all associations). There were significant trends for 25(OH)D to be higher in African and male subjects ($p<0.001$ for all comparisons).

Multivariate associations of 25(OH)D and PTH with components of the Met S are presented in table 5, with age, gender and BMI included as independent variables in all models. Addition of ethnicity to all the models demonstrated that Indian ethnicity was a positive determinant of glucose, triglyceride and HOMA levels but a negative determinant of diastolic and systolic blood pressure. Addition of 25(OH)D to these models had minimal effect on the beta values for ethnicity and in none of the models was 25(OH)D shown to be a determinant of any of the components of the metabolic syndrome, or HOMA. When PTH was added to each of the models, neither the ethnicity or 25(OH)D beta values were affected, however PTH was shown to be positively and significantly associated with systolic ($p = 0.018$) and diastolic ($p = 0.005$) blood pressures and waist circumference ($p<0.0001$) and negatively associated with HOMA ($p = 0.008$) levels.

Table 6. Multivariate logistic regression analysis for determining the effect of ethnicity, PTH and 25(OH)D on risk of metabolic syndrome.

Model numbers	Independent variables	Odds ratios	95% CIs	p-values
1	Ethnicity	2.24	1.57, 3.18	<0.0001
2	Ethnicity	2.29	1.60, 3.28	<0.0001
	Log 25(OH)D	1.15	0.78, 1.70	0.47
3	Ethnicity	2.27	1.56, 3.30	<0.0001
	Log 25(OH)D	1.25	0.67, 2.24	0.48
	Log PTH	2.48	1.01, 6.08	0.04

All models adjusted for age, gender and BMI, with metabolic syndrome as the dependent variable. Ethnicity, 25(OH)D and PTH were added in a forward, stepwise manner.
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Logistic regression was used to determine whether the higher prevalence of Met S in the Indian population was related to PTH or 25(OH)D levels. The data in Table 6 shows that after adjusting for age, gender and BMI Asian Indian ethnicity carries a 2.24 (95% CIs 1.57, 3.18; $p<0.0001$) increased risk of Met S compared to African subjects (model 1). Additional adjustment for 25(OH)D had no effect on the p-value (or odds ratio (OR)) for ethnicity and had no significant effect itself (model 2). The final addition of PTH to the logistic regression model (model 3) had no effect on the p-value or OR for ethnicity or 25(OH)D but did have a significant effect itself (OR = 2.48; 95% CIs 1.01, 6.08; $p = 0.04$).

In order to determine which individual components of the Met S are influenced by PTH to increase the risk of Met S, logistic regression analysis was performed. The results in Table 7 show that the increased risk for Met S conferred by PTH arises principally through its effect on systolic blood pressure and its positive association with waist circumference. This is demonstrated by the fact that adding these variables individually to the logistic regression model attenuates the p-value of the OR for PTH (see Table 7, models 6 and 7). Adding both these variables to the regression model at the same time attenuates the p-value for the PTH OR to a greater extent than adding each variable on its own (see Table 7, model 8). This suggests that the effects of each variable are additive and independent. Addition of the other metabolic syndrome components to the logistic regression model does not attenuate the p-value/OR for PTH and in some cases increases it. The exception is glucose, which does have a weak effect on the p-value for PTH, increasing it from 0.04 to 0.09 (see Table 7, model 4).

Discussion

In this study we investigated whether 25(OH)D or PTH is associated with the Met S or its components. Our results show a significant association of PTH, but not 25(OH)D, with the Met S. Higher PTH levels were associated with increased systolic and diastolic blood pressure and reduced insulin resistance as assessed by the HOMA index and an increased odds ratio for the Met S. Furthermore our results suggest that neither 25(OH)D nor PTH contribute to ethnic differences in the prevalence of Met S between African and Asian Indian subjects. There are no previous studies from sub-Saharan Africa that have examined the relationship between 25(OH)D, PTH and the Met S. Our results also showed lower levels of 25(OH)D in Asian Indians compared to

Table 7. Risk of metabolic syndrome due to PTH and the effects of adjusting for individual components of the metabolic syndrome on PTH odds ratios in a logistic regression analysis.

Model number	Independent variables	Odds ratios	95% CIs	p-values
1	Log PTH	2.48	1.01, 6.08	0.04
2	Log PTH	2.91	1.08, 7.83	0.03
	T riglyceride ≥1.7 mmol/L	11.9	7.19, 19.6	<0.0001
3	Log PTH	3.08	1.09, 8.76	0.03
	HDL-C <1.3 mmol/l (females) or <1.0 mmol/L (males)	13.3	8.41, 21.0	<0.0001
4	Log PTH	2.28	0.87, 5.97	0.09
	Glucose ≥5.6 mmol/L	9.58	5.90, 15.6	<0.0001
5	Log PTH	2.76	1.05, 7.24	0.04
	Diastolic bp≥85 mm/Hg	5.85	3.92, 8.73	<0.0001
6	Log PTH	1.76	0.66, 4.65	0.25
	Systolic bp≥130 mm/Hg	8.32	5.28, 13.1	<0.0001
7	Log PTH	2.03	0.82, 5.06	0.13
	Waist ≥94 or ≥90 cm for African or Indian males or ≥80 cm for females	9.69	4.55, 20.6	<0.0001
8	Log PTH	1.43	0.53, 3.85	0.48
	Systolic bp≥130 mm/Hg	8.46	5.32, 13.5	<0.0001
	Waist ≥94 or ≥90 cm for African or Indian males or ≥80 cm for females	10.3	4.66, 22.7	<0.0001

All models adjusted for age, gender, ethnicity, BMI and 25(OH)D with metabolic syndrome as dependent variable.
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Africans. Studies from India have also shown that this population tends to have poor vitamin D status [22,23].

Our observations of a lack of association of 25(OH)D with Met S are in line with those of Scragg et al. who observed no association between 25(OH)D status and type 2 diabetes in non-Hispanic black subjects but did observe a negative associations between 25(OH)D and risk of diabetes in Mexican Americans and non-Hispanic white populations [24]. Similarly, in a single study from India, Majumdar et al. showed that although 25(OH)D insufficiency was highly prevalent it was not associated with the Met S or insulin resistance [25].

The results of studies on the association of 25(OH)D and PTH with the Met S, components of the Met S or related disorders have been inconsistent. In a population based cross sectional study of US men and women over 20 years-of-age (NHANES III,1988–94) the prevalence of Met S and its components fell across increasing quartiles of 25(OH)D after adjustment for age, race, sex, income, lifestyle factors, calcium and energy intake. This study also showed that the odds ratio for Met S increased with increasing PTH in older men only [26]. In a study carried out in an aging European population, investigators showed that the risk for Met S decreased across increasing quintiles of 25(OH)D but could show no association of PTH with Met S [10]. Neither of these studies adjusted for BMI. However, Reis et al. [27] showed a lack of association between serum 25(OH)D and Met S in an adult Caucasian population. Our population, particularly the African subset, was not particularly vitamin D deficient overall (median 25(OH)D in Africans was 71 nmol/l) and 25(OH) D may be more strongly associated with Met S in a deficient population. However, this cannot explain the lack of association of 25(OH)D with Met S in the Asian Indian group, as serum 25(OH)D levels were significantly lower in this population compared to the African cohort.

A number of other studies have implicated PTH as being associated with the Met S rather than 25(OH)D [10,11]. Several lines of evidence support a role of PTH in increasing the risk of CVD. Thus, it has been associated with increased cardiovascular mortality in selected population groups [28,29,30] and with increased coronary heart disease in a younger group [31]. This increased risk may be mediated via its effects on blood pressure, insulin resistance, hyperglycaemia and low HDL-C [26,27]. In multivariate regression models we confirmed a positive relationship between PTH and blood pressure but could not verify any relationship between PTH and blood glucose or lipid levels, although significant associations were observed in univariate analyses. The negative relationship that we observed between PTH and the HOMA index was only observed after adjusting for confounding variables within a multivariate regression analysis. In a univariate analysis PTH correlated positively with HOMA. Derangements in glucose metabolism in primary hyperparathyroidism are well described with increased PTH increasing glucose intolerance [26,32,33,34]. A few studies have described a positive relationship between PTH and insulin resistance similar to our observation in univariate analyses. Some investigators have postulated that this relationship may be secondary to hypercalcaemia as it is absent in normocalcaemic subjects [35,36]. Frost et al. showed a significant negative association of PTH with insulin resistance in young men [37]. The negative relationship we have noted between PTH and HOMA in a multivariate analysis may be due to a number of reasons: ethnic differences, the fact that our subjects were all normocalcaemic or the adjustment for confounders. In addition to its possible effect on insulin sensitivity clinical evidence also supports a role of PTH in increasing blood pressure [13] and observational studies have linked elevated PTH levels to an increased risk of hypertension, left ventricular hypertrophy, and cardiovascular morbidity and mortality [30]. It is thought that PTH mediates its effect by directly increasing the

secretion of aldosterone from the adrenal glands and indirectly by activating the renin-angiotensin system [38].

Logistic regression models demonstrated that the increased risk of Met S in subjects with higher PTH levels is largely due to the positive and independent relationships of systolic blood pressure and waist circumference with PTH. Previous studies have also shown a positive association of PTH with obesity [30,39]. Furthermore body weight changes correlate positively with changes in serum PTH levels [40,41] suggesting that obesity may be causative for hyperparathyroidism. There are no studies in the literature to show that PTH is secreted by adipocytes but there are studies that demonstrate that PTH does modify adipocyte function. These effects include the inhibition of adipocyte lipoprotein lipase activity [42] and the attenuation of insulin signalling [43]. However, both these actions of PTH would limit rather than augment triglyceride deposition within adipose tissue. It is possible that increased adiposity may lead to greater production of PTH by the parathyroid, and it is interesting to note that studies have shown a positive correlation between serum leptin and PTH concentrations [44,45].

Our data show that Asian Indian ethnicity is associated with increased risk for Met S and most of its components. As a group, Asian Indians tend to be insulin resistant and at high risk for diabetes and premature coronary heart disease when compared to other ethnic groups [46] and this is partly explained by high visceral fat content [47]. In the present study ethnic differences in the levels of Met S-related metabolic variables and the prevalence of Met S were investigated using multiple regression and logistic regression analyses and were found not to be related to ethnic differences in PTH or 25(OH)D levels.

This study is limited by the cross-sectional nature of the data, and as such we cannot draw any conclusions about causality. Another limitation is that blood samples were collected over several months, and vitamin D is known to display seasonal variation. Also, study subjects were not selected randomly and this may have introduced some level of selection bias. Finally, 25(OH)D levels were not measured using the reference method of liquid chromatography-tandem mass spectrometry (LC-MS/MS), however the technique used in this study (HPLC) has been shown to correlate well with the LC-MS/MS method [48].

In conclusion, PTH but not 25(OH)D is associated with the Met S in our African and Asian Indian populations. The PTH effect is largely via its impact on blood pressure and its relationship with waist circumference. The relationship between PTH and insulin resistance needs to be investigated further, as does the mechanism responsible for causing serum PTH levels to rise with increased adiposity.

Author Contributions

Conceived and designed the experiments: JAG SAN NJC. Performed the experiments: JAG. Analyzed the data: JAG NJC HEvD. Contributed reagents/materials/analysis tools: JAG. Wrote the paper: JAG NJC.

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