FACTORS INFLUENCING THE VITAMIN D STATUS OF ADOLESCENTS IN JOHANNESBURG, AND ITS EFFECTS ON BODY COMPOSITION

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

January 2021

DECLARATION

I Machuene Ananias Poopedi, declare that this thesis is my own original work. It is being submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted previously for any degree or examination at any other University.

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(Signature of the candidate)

__13th ____day of January_____2021__in Parktown, Johannesburg

DEDICATION

To my parents

My Late Mother, Chuene Abrina Poopedi (Nèè Tsiri) [Mme Sekiele], 1945 -2016 RIP, your departure was sudden and unexpected. This was a painful separation and you will remain a missing link in my life.

My Father Mathuku Petrus Poopedi [Bra-Peter]

I know it took a while for me to complete this project, now it's time to celebrate. Thanks a lot for your encouragement, patience and for supporting me in all academic endeavours that I wanted to pursue in my life. "Magoro ke Matshoshetjani" **Piet Metse Poopedi**, 1896 – 1991 RIP.

"It always seems impossible until it is done"

The Late President Nelson Rolihlahla Mandela, 1918 – 2013.

"Aha Dhalibunga"

THE RESEARCH FOR THIS THESIS RESULTED IN THE FOLLOWING PRESENTATIONS

POSTER PRESENTATIONS

1. **Poopedi MA,** Norris SA, Pettifor JM, **Factors influencing the vitamin D status of 11 year old urban South African children.** Presented at the 5th International Congress for Children Bone Health (ICCBH), Cambridge, United Kingdom (UK) 2009.

ORAL PRESENTATIONS

1. **Poopedi MA,** Norris SA, Pettifor JM, **Gender and ethnic differences in vitamin D status and biochemical bone markers in children.** Presented at the 13th conference of the National Osteoporosis Foundation of South Africa (NOFSA) Cape Town, 2008.
2. **Poopedi MA,** Norris SA, Pettifor JM, **Vitamin D status in South African children**, Presented at the SA Nutrition Congress, Evidence based nutrition leading the way in Innovation, University of Pretoria, 2008.
3. **Poopedi MA,** Norris SA, Pettifor JM, **Gender and ethnic differences in vitamin D status, bone turnover markers and body composition in children**, Presented at the University of the Witwatersrand Faculty of Health Sciences Research Day, 2008.
4. **Poopedi MA,** Norris SA, Pettifor JM, **Vitamin D status in children: Analysis of year 10, 11 and 12 data and its longitudinal assessment,** Presented at the MRC Review, Developmental Pathways for Health Research Unit, Chris Hani Baragwanath Hospital, 2010.
5. **Poopedi MA,** Norris SA, Pettifor JM, **Longitudinal assessment of Vitamin D status in urban South African children,** Presented at the 13th Congress of the National Osteoporosis Foundation of South Africa (NOFSA), Durban, 2010.
6. **Poopedi MA,** Norris SA, Pettifor JM, **The association between vitamin D status and body composition, serum lipids and insulin resistance (HOMA-IR) in urban 12 year old South African children,** Presented at the 15th Congress of the National Osteoporosis Foundation of South Africa (NOFSA), Cape Town, 2012.
7. **Poopedi MA,** Norris SA, Pettifor JM, **Does vitamin D track through adolescence?** Presented at the 16th Conference of the National Osteoporosis Foundation of South Africa (NOFSA), Johannesburg, 2014.

PUBLICATIONS

Paper 1 – Poopedi MA, Norris SA, Pettifor JM, **Factors influencing the vitamin D status of 10-year-old urban South African children.** Public Health Nutrition 2011, 14(2): 334-339.

Student's contribution to the paper

The student was responsible for running of the samples for 25(OH)D, initial conceptualization and design of the study, data analysis and interpretation of the data, drafted the initial manuscript, and approved the final manuscript as submitted.

Paper 2 – Poopedi MA, Norris SA, Micklesfield L, Pettifor JM, **Does vitamin D track through adolescence?** American Journal of Clinical Nutrition. 2015,102 (5):1025-1029.

Student's contribution to the paper

The student was responsible for running of the samples for 25(OH)D, initial conceptualization and design of the study, data analysis and interpretation of the data, drafted the initial manuscript, and approved the final manuscript as submitted.

Paper 3 - Poopedi MA, Norris SA, Pettifor JM, **Is vitamin D status associated with non-communicable disease risk in children?** A cohort study. (Under review: South African Journal of Clinical Nutrition).

Student's contribution to the paper

The student was responsible for running of the samples for 25(OH)D and lipid profile, initial conceptualization and design of the study, data analysis and interpretation of the data, drafted the initial manuscript.

ACKNOWLEDGEMENTS

My Supervisors

Professors JM Pettifor and SA Norris

I don't have proper English words to express my sincere appreciation, and most importantly your patience in guiding me in the process of familiarization of my research project and writing this thesis. **You literally held my hand.** Thanks for believing in me and mentoring me on the aspects of bone and mineral research and statistical concepts. I will not mind to choose the both of you as my supervisors in my next life. Ke leboga Kudu Kudu hle!!!

To my former employer at Wits University and present employer at Polokwane Hospital

Professors MT Ramokgopa (Academic Head of Orthopaedics), Professor AA Aden (Deputy Academic Head), and Dr FLM Hyera (Public Health Medicine, Polokwane Hospital and University of Limpopo – HOD).

Thanks for making a suitable environment for me to work and study in the Orthopaedic and Public Health Medicine departments.

Birth to Twenty and DPHRU staff members, past and present

Thanks a lot for your dedication in data collection and storage, your efforts haven't gone unnoticed.

Study participants

This project was not going to take off without you coming voluntarily to our site, from the bottom of my heart I sincerely **Thank You!!!**

My Brothers

Washington (**Bigs-Bazooka**) – Mokone di tsene kamoka ka 'shakeng.

Ezekiel (**Bassie**), Gideon, – I know how much you wanted me to complete this project, now it's time to celebrate.

My Sisters (Cheer Leaders)

Mashita Salome Poopedi- Mothokoa you don't have to stress now, Ke feditje bjalo.

Metse Petunia Thebe (**Nèè Poopedi**) –Ke a tseba gore ke kgale le letile, Mothokoa.

Mmapadi Selina Matsweneng (**Nèè Poopedi**) – Thanks for always willing to carry my bags. Much Appreciated!!!

Ragwane Lazy (Mlaza)

Now that the project is complete, I hope you will give me the permission to have a glass of wine after dinner.

Buti Mmapadi (Mr Mafisa)

Thanks for playing the Big-Brother role in my family tree. Remember this academic journey started in 1994 and is still continuing…

Mr Saki Maupye's family

Bakone ba Matlala ke lebogile thego ya lena. It's time to give thanks and praise to the Lord and let's celebrate.

Mr Joseph Wamkou (Health Economist)

Bwana Wamukuo, Thanks for helping me with my manuscript page arrangement. Asante Sana.

Hendrick and Moses Tsiri (Cousins)

You are more than friends to me. Motswala Drika, you always say ke mmila o mo sesane… bjalo re nale sekopodi ka gae.

My wife

Kgomotso Poopedi (**Nèè**) Lebogo, Thanks for your patience and support throughout my academic journey. Much Love!!!

Last but not least Lord Almighty

Thanks for giving me the courage and the wisdom to soldier on with this project because it wasn't easy….

"Nothing is so fatiguing as the eternal hanging on of an uncompleted task" **William James**

Abstract

Introduction

Sunlight is nature's gift to human beings in the cutaneous production of vitamin D. However, one of the challenges faced by public health in the majority of countries across the globe is hypovitaminosis D across all ages. Factors such as skin pigmentation, cloud cover, air pollution, increase in body fat content, an indoor lifestyle, and a lack of exposure of the skin to sunlight due to clothes covering the body, have been reported as responsible for hypovitaminosis D. The effects of this are rickets in children, osteomalacia in adults and osteoporosis in the elderly. Recent reports have found that vitamin D has functions in addition to bone health, including immune system benefits as well as cell proliferation, and differentiation. Furthermore, vitamin D status has been associated with a variety of diseases. However, the majority of reports have assessed vitamin D status by measuring it once, suggesting that vitamin D status is constant over the years, but this is far from the truth. Hence some researchers have suggested that vitamin D status should be measured longitudinally because of the factors involved. One challenging issue has been vitamin D laboratory assays that were reported to be inconsistent, which has led the researchers to call for standardization. There has been a lack of consensus among researchers in defining vitamin D status, thus some researchers' definition covers bone health and others use a definition which includes other organs beyond bone health. Studies of vitamin D status in children, its tracking over a period of time and its association with non-calcaemic diseases are a handful. Hence, the present study was aimed at assessing factors influencing vitamin D status, its tracking over 10 years and its effects on body composition in adolescents living in Johannesburg.

Methods

The group of children who formed the basis of my studies are the Bone Health sub-cohort of the Birth to Twenty cohort. This is a longitudinal study of child health and development, which has followed the development of 3273 children in the Greater Johannesburg area of South Africa since their birth in 1990. For anthropometric measurements and body composition, height was measured in millimetres (mm) using a wall-mounted stadiometer (Holtain, UK) and weight in kilograms (kg) using a digital electronic instrument (Dismed, USA). Both instruments were regularly calibrated and subjects wore minimal clothing when being weighed. Body mass index

(BMI) was calculated as weight (in kg)/height^2 (in m). Total fat mass and lean mass were measured by DXA, using Hologic QDR 4500 (Hologic Inc, Bedford, MA, USA).

Factors influencing the vitamin D status of 10-year-old urban South African children were studied. Vitamin D status was assessed in 385 subjects because there were blood samples available. Furthermore, vitamin D tracking over a ten year period was conducted in 504 subjects; of these 99 subjects met the criteria of being available at the study site for a minimum period of 3 or more years. For an assessment of the association of the vitamin D status with non-communicable disease risk in children, variables were measured in years 11, 12 13, 15 and 18-20 years. The variables assessed included anthropometry (weight, height and BMI), blood pressure (SBP and DBP) and 25(OH)D levels. The lipid levels (TC, Trig, HDL-C, and LDL-C) were only measured in year 12 and 18-20 year old participants. For age related changes between the two time points, year 12 participants (N = 261) were matched with those available in year 18-20 (N = 368) resulting in 200 in each group. The data for longitudinal analysis using the GEE comprised of the following groups of subjects, namely Year 11 (N = 288), Year 12 (N = 253), Year 13 (N = 292), Year 15 (N = 238) and Year 18-20 (N = 368).

Results

In 10-year-old children, white children had significantly higher 25(OH)D than their black peers (120 ± 14.7 nmol/L and 93 ± 13.4 nmol/L respectively; p = 0.0001). Seasonal variations in 25(OH)D levels were found only in the white group of children, with 25(OH)D levels being significantly higher in white than black children during the autumn and summer months (p = 0.01 and p = 0.0001 respectively).

During vitamin D tracking, no significant correlation between 25(OH)D in the earlier and later years of adolescence was found, although there were significant correlations between year 11 and year 13 ($r = 0.71$; $p < 0.0001$), and between years 15, 17 and 20 ($r \geq 0.65$; $p < 0.0001$). The percentage of adolescents whose 25(OH)D concentration changed by > 20 nmol/L from year 11 was calculated for all age groups: 12% of the cohort had a change of > 20nmol/L at 13 years of age compared to 46% at 20 years of age. Just more than one-half (53%) of the cohort changed their category of vitamin D status between the ages of 11 and 20 years; one-third of adolescents changed their vitamin D status from being replete to insufficient over the same period.

In assessing the association of vitamin D status with non-communicable disease risk, there were significant increases of mean BMI, BP and decreases in 25(OH)D levels with age (all p-values < 0.0001). In females, systolic BP was significantly higher in older participants (18-20 years of age) than younger participants (12 years of age), but 25(OH)D was significantly higher in younger than older participants. In males, there was a significant increase in BP in participants between the ages of 12 years and 18-20 years. 25(OH)D, total cholesterol (TC), and low density lipoprotein (LDL-C) were significantly lower in 18-20 year old participants compared to 12 year old participants. Longitudinally, 25(OH)D was only associated inversely with LDL-C.

Conclusion

The majority of children aged 10 years (74%) had "sufficient" vitamin D status (25(OH)D > 75 nmol/L). Thus routine vitamin D supplementation is not warranted in children residing in the Johannesburg area but it is unknown whether similar results would be obtained in regions that are further south (such as Cape Town) or in inner city children living in crowded high-rise buildings. In the longitudinal study of vitamin D status, there was no long-term association between values obtained in the early years of adolescence and those of the later years. These results thus question the validity of those studies that have assessed the association of vitamin D status with disease outcomes, but in which vitamin D status was only measured once during the study. Finally, there was an increase in BMI and BP, and a decrease in 25(OH)D in adolescents followed longitudinally over a period of 10 years. After controlling for covariates, 25(OH)D was only significantly negatively associated with LDL-C, which suggests that vitamin D status might be associated positively with favourable lipid profiles in children and adolescents.

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$1,25(OH)_2D$	1,25-Dihydroxyvitamin D
7DHC	7 Dehydrocholesterol
25(OH)D	25-Hydroxyvitamin D
25-OHase	Vitamin D-25-hydroxylase (CYP2R1)
AODM	Adult Onset Diabetes Mellitus
APC	Antigen Presenting Cells
ASCVD	Atherosclerotic Cardiovascular Disease
C	Carbon
CaBP	Calcium Binding Protein
CBT	Childhood Brain Tumour
CDC	Centre for Disease Control and Prevention
CD4	Cluster of Differentiation
cDNA	Single Stranded Deoxyribonucleic Acid
CHD	Coronary Heart Disease
CI	Confidence Interval
CLIA	Chemiluminescence Immunoassay
CPBA	Competitive Protein Binding Assays
CYP	Cytochrome P450
CYP24A1	Vitamin D 24-Hydroxylase
CYP27A1	Sterol 27 Hydroxylase
CYP27B1	1α-Hydroxylase

CYP2R1	Vitamin D-25-Hydroxylase
DBP	Vitamin D Binding Protein
DC	Dendritic Cells
Deqas	Vitamin D External Quality Assessment Scheme
EDSS	Expanded Disability Status Scale
FEV	Forced Respiratory Volume
FBG	Fasting Plasma Glucose
FGF 23	Fibroblast Growth Factor 23
GIT	Gastrointestinal Tract
GCPL	Good Clinical Practice Laboratory
GWAS	Genome Wide Association Studies
HAART	Highly Active Antiretroviral Therapy
HBP	High Blood Pressure
HDL-C	High Density Lipoprotein Cholesterol
HMG-CoA	Hydroxymethylglutaryl-Coenzyme A
HPLC	High Performance Liquid Chromatography
HPLP-APCI-MS	High Performance Liquid Chromatography-Atmospheric Chemical Ionization-Mass Spectrometry
IFN-ϒ	Interferon Gamma
Ig	Immunoglobulin
IOM	Institute of Medicine
IUPAC	International Union of Pure and Applied Chemistry
KNHANES	Korea National Health and Nutrition Examination Survey
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LDL-C	Low Density Lipoprotein Cholesterol
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis

NHANES	National Health and Nutrition Examination Survey
NIDDM	Non-Insulin Depended Diabetes Mellitus
NIST	National Institute of Standard and Technology
NKC	Natural Killer Cells
Nm	Nanometer
OR	Odds Ratio
PTB	Pulmonary Tuberculosis
PTH	Parathyroid Hormone
RA	Rheumatoid Arthritis
RANK	Receptor Activator of Nuclear Factor Kappa-β
RANKL	Receptor Activator of Nuclear Factor Kappa-β Ligand
RCT	Randomised Clinical Trials
SP	Sun Protector
SZA	Solar Zenith Angle
TB	Tuberculosis
TC	Total Cholesterol
TGF-β	Transformation Growth Factor
Th-2	T-helper Cells
TLR	Toll-like Receptors
TNFα	Tumour Necrosis Factor Alpha
TNFβ	Tumour Necrosis Factor Beta
Trig	Triglycerides
UK	United Kingdom
URTI	Upper Respiratory Tract Infection
US	United States
UTI	Urinary Track Infection
UVB	Ultraviolet Beta
VDSP	Vitamin D Standard Program
VDR	Vitamin D Receptor
WBS	Williams Beuner Syndrome

PART 1: INTRODUCTION, LITERATURE REVIEW AND METHODOLOGY

CHAPTER 1: INTRODUCTION

1.1 Rationale

Since the beginning of human and vertebrate existence over 160000 years ago, vitamin D – the sunshine vitamin - has been acquired through the exposure of the skin to solar radiation (wavelength, 290-315 nm). This leads to the conversion of 7-dehydrocholesterol to pre-vitamin D_3 in the skin (Holick, 1981, Holick, 1989), which then undergoes thermal isomerization to cholecalciferol or vitamin D_3. Synthesized vitamin D in the skin formed via direct sunlight may provide 80 to 90% of the human body's requirements, with the remaining proportion normally acquired through dietary sources (German, 2012). Vitamin D as a nutrient is obtained from irradiated mushrooms, mackerel, egg yolks, cod-liver oil, oily fish (salmon, sardines, herring, anchovies, trout) (Holick, 2004b, Lips, 2006, O'Connor et al., 2013) and fortified foods such as milk, margarine, cheeses, yoghurts, and orange juice (Holick, 2008).

Physiologically, vitamin D obtained from diet (vitamin D_2 or D_3) and sunlight (vitamin D_3) is hydroxylated to 25-hydroxyvitamin D (25(OH)D) in the liver. It is activated by being converted in the kidneys to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which stimulates intestinal calcium and phosphorus absorption, facilitating the development, growth, and maintenance of skeletal health (Holick, 2003, Lanske and Razzaque, 2007, Wacker and Holick, 2013). Vitamin D metabolites differ in terms of their half-lives in blood, with 25(OH)D and $1,25(OH)_2D$ having half-lives of approximately 25 days and 7 hours respectively. 25-Hydroxyvitamin D is an inactive form of the vitamin, whose serum levels reflect the vitamin D status of an individual. The active form of the vitamin ($1,25(OH)_2D$) is tightly regulated and its circulating levels provide limited information about vitamin D nutritional status. Its measurement is useful in the disorders of 1α-hydroxylation in the kidney, such as in vitamin D dependent rickets Type I, chronic renal failure and conditions associated with excess serum levels of fibroblast growth factor 23 (FGF23). Over the last 30 or more years, vitamin D has been found to be involved in many other functions, including cell differentiation, function, and proliferation (Moukayed and Grant, 2013) and has also been reported to be related to diseases that are non-skeletal in nature such as asthma (Bose et al., 2013, Montero-Arias et al., 2013), tuberculosis (TB) (Hong et al., 2014, Barr et al., 2017), type I and II diabetes mellitus (Holick, 2004b, Boucher, 2012), autoimmune diseases, cardiovascular disease, cancers (Holick, 2004a), and multiple sclerosis (Salzer et al., 2012, Bermúdez-Morales et al., 2017).

A major issue of contention is how vitamin D status is defined in terms of deficiency, insufficiency and sufficiency (replete). At present there are several recommendations [such as the United States (US) Endocrine Society, US Institute of Medicine (IOM) and from the United Kingdom (UK)] which have defined vitamin D status (Pearce and Cheetham, 2010, Ross et al., 2011b, Holick et al., 2011, Rosen et al., 2012, Balasubramanian et al., 2013, Manson et al., 2016, Prentice, 2016, Scientific Advisory Committee on Nutrition, 2016). Irrespective of which definition is used, the prevalence of hypovitaminosis D is high among most world populations (Mithal et al., 2009, Bassil et al., 2013). Vitamin D deficiency has been reported to occur worldwide in the young, adults and elderly (Bhattoa et al., 2004, Harinarayan et al., 2007, Van Schoor and Lips, 2011, Bassil et al., 2013). Vitamin D deficiency also poses a major challenge to public health through its involvement in physiological pathways that are beyond bone function (Jun et al., 2017, Looman et al., 2017).

Vitamin D status is influenced by factors that affect skin production or vitamin D absorption in the intestines. Skin synthesis is influenced by factors such as UV radiation reaching the skin, season of the year (Harris and Dawson-Hughes, 1998, Looker et al., 2002, Levis et al., 2005), latitude and atmospheric pollution (Agarwal et al., 2002), melanin content of the skin (Harris, 2006), and the surface area of skin exposed to sunlight (El-Sonbaty and Abdul-Ghaffar, 1996, Allali et al., 2006, Harris, 2006). These factors affect populations across all age ranges. Although religion per se does not influence vitamin D, the social and dress customs associated with a religion may influence vitamin D status (Taha et al., 1984, Fonseca et al., 1984, Lips, 2007, Alsuwaida et al., 2013). Because of these factors, vitamin D status tracking over a period of time poses a challenge. A handful of researchers are suggesting that it is difficult to assess the habitual vitamin D status of an individual by measuring it once only, due to variations in the factors influencing vitamin D status over time (Moon et al., 2015, Zhu et al., 2017).

In a situation where there is excessive sunlight exposure, sunlight degrades previtamin D_3 and vitamin D_3 to inactive photoproducts, thus preventing excessive production of vitamin D in the skin (Luxwolda et al., 2012, Grober et al., 2013). Vitamin D toxicity is only encountered when high vitamin D doses are used for treatment, supplementation or to fortify foodstuffs, which are usually consumed over a prolonged period of time. Vitamin D toxicity can also happen when a single megadose is ingested resulting in a large increase in circulating 25(OH)D levels (Pettifor et al., 1995, Vieth, 1999).

Measurement of blood samples for 25(OH)D in the laboratory is achieved by a variety of assays including chemiluminescence immunoassays (CLIA), radioimmunoassays (RIA), high-performance liquid chromatography (HPLC), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Atef, 2018). Binding assays like competitive protein binding assays (CPBA) have been reported to underestimate 25(OH)D at low levels and overestimate it at high levels (Hollis, 2004). HPLC is a technique which is very stable and measures 25(OH)D_2 and 25(OH)D_3 separately, but it requires a larger sample and a preparation step before chromatography. In some instances, the assay is subjected to interferences from other compounds. LC-MS/MS has the advantage of being accurate, precise and specific for the quantitation of 25(OH)D_2, 25(OH)D_3 and epi-25(OH)D_3 in serum samples (Schleicher et al., 2011). However, some researchers have found the presence of 25(OH)D_3 C-$_3$ epimers in pediatric specimens which may overestimate 25(OH)D concentrations (van den Ouweland et al., 2011, Yazdanpanah et al., 2013). This vitamin D metabolite (25(OH)D_3 C-$_3$ epimers) has been shown to be present in infants of up to 1 year of age and it may contribute up to 61% of total 25(OH)D (Yazdanpanah et al., 2013).

Serum 3-epi-25(OH)D can also be erroneously included as 25(OH)D if immunoassays display cross-reactivity to the C_3-epimer or if HPLC or LC-MS/MS methods do not resolve both vitamin D compounds (Ooms et al., 2016). In a study on 204 twins from the Swedish Twin registry, researchers found a major disagreement between 25-hydroxyvitamin D levels as determined by different assay techniques (Snellman et al., 2010). Mean 25-hydroxyvitamin D levels were highest using the high-pressure liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (HPLC-APCI-MS) technique (85 nmol/L, 95% CI 81–89), intermediate for RIA (70 nmol/L, 95% CI 66–74) and lowest with CLIA (60 nmol/L, 95% CI 56–64). Furthermore, using a 25(OH)D value of 50 nmol/L as the lower cut-off for vitamin D sufficiency, 8% of the subjects were insufficient using HPLC-APCI-MS, 22% with RIA and 43% by CLIA. Hence, researchers have called for the vitamin D assay methodology to be standardized (Binkley et al., 2004). However, HPLC and LC-MS/MS methods are still the preferred techniques for quantitatively measuring 25(OH)D levels (Holick, 2009b, Schleicher et al., 2011, Farrell et al., 2012).

South Africa has sunlight in abundance, but only a handful of studies on vitamin D status have been reported. However, these are cross-sectional studies and vitamin D status was only

measured once during the study (Daniels et al., 1997, Kruger et al., 2011, Martineau et al., 2011b, Velaphi, 2017). Vitamin D status in SA has been reported to be generally sufficient (25(OH)D > 50 nmol/L) in children and adults but deficient in elderly and adolescents with protracted binge drinking (Naude et al., 2012, Norval et al., 2016). In order to extend the existing research, the present study assessed the vitamin D status of adolescents, its tracking over a period of ten years and the relationship between vitamin D status and body composition.

1.2 Study Aims

To assess the vitamin D status and its tracking patterns in adolescence.

To assess the impact of vitamin D status on body composition in adolescence.

1.3 Study Objectives

To evaluate 25(OH)D status based on ethnicity and season of the year.

To measure 25(OH)D levels in a longitudinal study over 6-8 years and to evaluate the tracking of vitamin D status with age and time.

To determine the impact of vitamin D status on body composition in adolescence.

To determine the impact of vitamin D on non-communicable disease risk in children and adolescents.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 Vitamin D nomenclature

The naming of vitamin D precursors and metabolites is according to the International Union of Pure and Applied Chemistry (IUPAC), for defining rules for the nomenclature of vitamins (Table 2.1). The naming was established in 1960 and revised in 1966 as follows; 7-dehydrocholesterol, cholecalciferol, ergocalciferol, calcidiol and calcitriol represent pro-vitamin D_3, vitamin D_3, vitamin D_2, 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D respectively.

Table 2.1: Nomenclature of vitamin D precursors and metabolites.

Common Name	Clinical Name	Abbreviation	Location or activity
7-dehydrocholesterol	Pro-vitamin D_3	7DHC	Lipid cell membrane.
Cholecalciferol	vitamin D_3		Photosynthesized in the skin or available in diet.
Ergocalciferol	vitamin D_2		Obtained from diet.
Calcidiol	25-hydroxyvitamin D	25(OH)D	Reflects quantitative vitamin D status.
Calcitriol	1,25-dihydroxyvitamin D	$1,25(OH)_2D$	Active form of vitamin D and tightly regulated.

Source: Alshahrani and Aljohani, 2013.

Pro-vitamin D_3 is found in lipid cell membranes and through photolysis and thermal isomerization is converted to vitamin D_3 in the skin. Vitamin D_2, formed by the photolysis of ergosterol, is obtained in the diet (Figure 2.1, page 8). The combined concentrations of $25(OH)D_2$ and $25(OH)D_3$ (denoted by 25(OH)D without a subscript) in serum reflect the overall vitamin status obtained from both diet and sunlight.

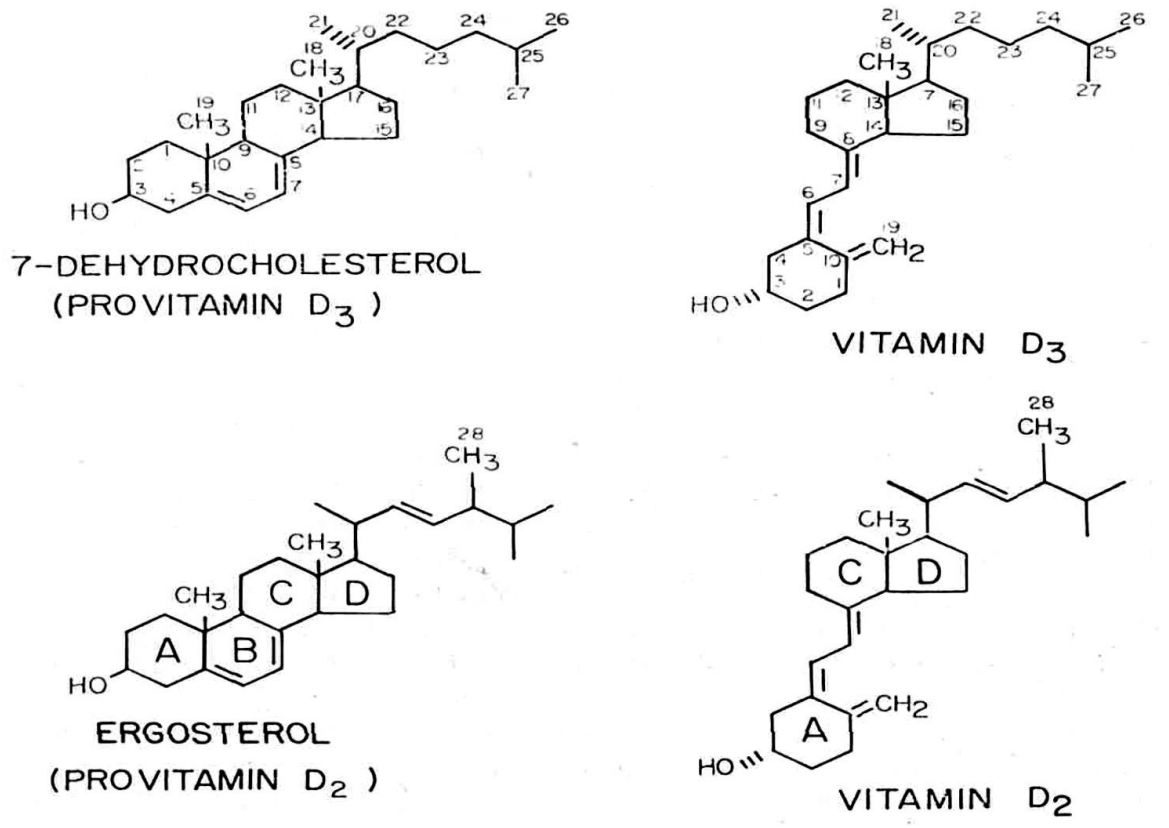


Figure 2.1: Biochemical structure of vitamin D and its precursors. Source: Holick, 1981.

2.2 Vitamin D metabolism

Vitamin D has been called 'the sunshine vitamin' because of the dependence of its production in the skin on ultraviolet radiation from the sun (Holick, 1981). During this process, 7-DHC undergoes the process of photochemical activation in the stratum germinativum by absorption of ultraviolet light from the sun and is converted to previtamin D_3 (Parfitt et al., 1982, Holick, 2007b, Vidailhet et al., 2012). Previtamin D_3 is immediately converted to vitamin D_3 through a temperature-dependent process. Excessive exposure to sunlight degrades previtamin D_3 and vitamin D_3 into inactive photoproducts (Holick, 2007b).

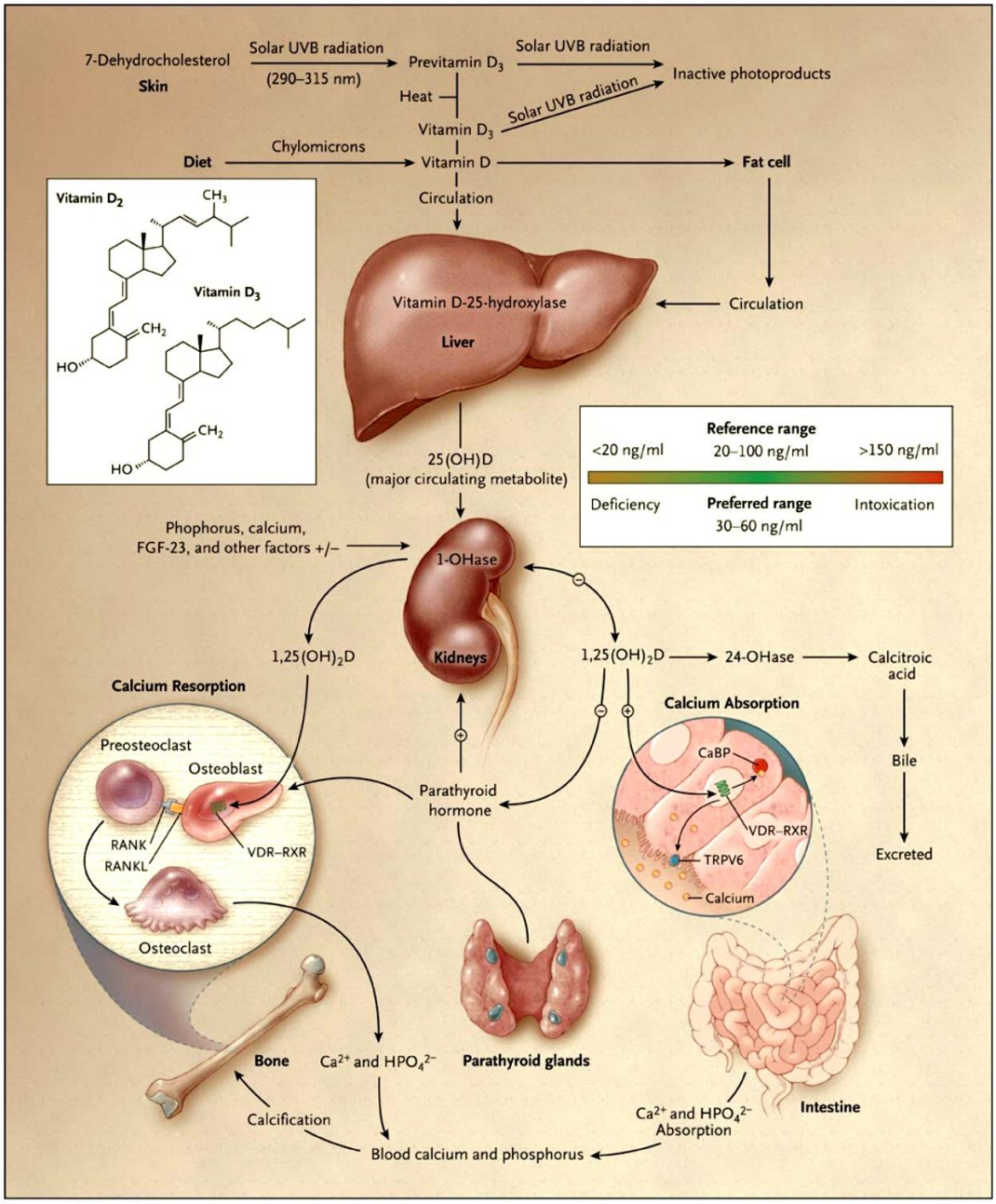


Figure 2.2: Synthesis and metabolism of vitamin D. Source: Holick, 2007b.

Figure 2.2 shows the two main sources of vitamin D in the human body. The overall body store of vitamin D depends on cutaneous production as well as on dietary intake.

The vitamin D pathway is via the skin and gastrointestinal tract (GIT). The main vitamin D form is through cutaneous synthesis, in which vitamin D production is solely depended on the incident angle of the sun, latitude, season and time of the day (Gómez-Alonso et al., 2003). The precursor

molecule which is pre-vitamin D is synthesized from 7-DHC in the epidermis and dermis during exposure to ultraviolet rays (290 – 315 nm). The process is temperature-dependent and is not regulated by enzymes, which result in subsequence transformation into vitamin D and binding to vitamin D-binding protein (DBP). Vitamin D is then delivered to the liver and metabolized to 25(OH)D, through an enzymatic process involving 25-hydroxylase (CYP2R1). The 25(OH)D undergoes a second hydroxylation in the kidney, and to a lesser extent in other tissues, by 1-alpha hydroxylase (CYP27B1) enzymatic activity, to become 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (Gómez-Alonso et al., 2003). In the GIT, vitamin D_2 and vitamin D_3 from dietary sources are incorporated into chylomicrons and transported by the lymphatic system into the venous blood circulation. Vitamin D formed in the skin or ingested in the diet can be stored in fat and muscle cells, from which it can be released in blood circulation. Vitamin D in the circulation is bound to the vitamin D–binding protein, which transports it to the liver, for the conversion by vitamin D-25-hydroxylase to 25(OH)D. This form of vitamin D is biologically inactive and must be converted in the kidneys by 25-hydroxyvitamin D-1αhydroxylase (1-OHase) to the biologically active form (1,25-dihydroxyvitamin D). Serum phosphorus, calcium, fibroblast growth factor 23 (FGF-23), and other factors can either increase or decrease the renal production of $1,25(OH)_2D$. 1,25(OH)2D decreases its own synthesis through negative feedback and decreases the synthesis and secretion of parathyroid hormone by the parathyroid glands. $1,25(OH)_2D$ increases the expression of 25-hydroxyvitamin D-24- hydroxylase (24-OHase) to catabolize $1,25(OH)_2D$ to the water-soluble, biologically inactive calcitroic acid, which is excreted in the bile (Holick, 2007b).

$1,25(OH)_2D$ enhances intestinal calcium absorption in the small intestine by stimulating the expression of the epithelial calcium channels and calbindin 9K (calcium-binding protein; CaBP). It also increases calcium reabsorption in the kidney and osteoclastic activity in bone. $1,25(OH)_2D$ also binds to its receptor in osteoblasts, causing an increase in the expression of receptor activator of nuclear factor kappa-B ligand (RANKL). Its receptor RANK on the preosteoclast binds RANKL, which induces the preosteoclast to become a mature osteoclast. The mature osteoclast removes calcium and phosphorus from bone to maintain blood calcium and phosphorus levels (Holick, 2008).

2.3 Classic and non-classic roles of vitamin D

As previously discussed, the classical role of vitamin D in humans is to increase the absorption of calcium and phosphate from the GIT for the mineralization of the skeleton (Khazai et al., 2008) and also to maintain the extracellular fluid concentrations of calcium and phosphorus within the normal range (Favus et al., 2006). Furthermore, non-classical roles for vitamin D have been investigated for the past several decades. VDRs are reported to be found in many tissues other than bone and small intestine such as in the immune system, prostate, brain and more (Khazai et al., 2008, Cavalier et al., 2009). It is suggested that non-bone related diseases such as diabetes mellitus, multiple sclerosis and some cancers may be associated with vitamin D deficiency (Hollis et al., 2007, Kimball et al., 2008, Zhang and Naughton, 2010, Zittermann et al., 2016).

2.4 History and evolutionary perspective of vitamin D

Researchers have suggested that vitamin D deficiency became a public health problem at the time of the industrial revolution. The effects of the industrial revolution, resulting in coal burning causing a pall of air pollution, were felt by northern European countries in the 18th and 19th centuries. The majority of buildings were in close proximity to each other, with the consequence that sunlight and UV radiation did not reach the narrow streets. This lack of sunlight led to a bone deforming disease called rickets in children, that had devastating health consequences (Holick, 2006). Rickets was initially regarded as a disease affecting rich people who lived in the city centres, rather than the poor who lived in rural areas of the country (Hess and Weinstock, 1924). Table 2.2 (page 12) shows the sequence of events leading to the discovery of vitamin D, as well as subsequent investigations into vitamin D. Milestones in vitamin D research are shown as well as the contributions of the scientists who participated in the research.

Table 2.2: Historical perspective of the discovery of vitamin D and the milestones in vitamin D research.

Sequence of events in the discovery and study of vitamin D	**Researchers involved in the discovery and study of vitamin D and bone deforming diseases.** The studies below were cited by Wacker and Holick, 2013.
Detailed description of the clinical features of rickets in England in the middle of the 17th century. Inadequate access to daily sunlight in Europe has led to the emergence of bone-deforming disease in children, which was endemic in Northern Europe.	Glisson, 1650.
Change in lifestyle (not being exposed to sunlight) and migration into the city during the industrial revolution (1760-1840) in Northern Europe led to rickets. This was because sunlight was restricted from reaching the skin surface as buildings were built in close proximity to each other and because of atmospheric pollution resulting from the combustion of coal.	Sniadecki and Sniadecki, 1939.
In 1890, a researcher recognized that children who lived in the industrialized cities of Great Britain were at high risk of developing rickets. This was in contrast to children living on the periphery of the cities who were free of rickets. The researcher concluded that sunbathing could prevent rickets and proposed that some type of sunshine recorder should be developed to measure the bone-healing properties of the sun.	Palm, 1890.

As years passed in search of a cure, European populations were still experiencing rickets, osteomalacia, and osteoporosis. In 1919 Huldschinsky, using a mercury arc lamp, reported that rachitic children exposed to UV light showed a dramatic radiological improvement after several months.	Huldschinsky, 1919.
In another investigation, researchers showed that exposure of rachitic children to sunlight on the rooftop of the New York City Hospital resulted in a significant radiologic improvement in the children's rickets.	Hess and Gutman, 1921. Maughan, 1928.
At the turn of the 20th century, studies reported that 80 – 90% of children in Northern Europe and Northern US had evidence of rickets. Researchers exposed various foodstuffs, including cottonseed oil, corn oil and milk to UVB radiation and showed that this process imparted antirachitic activity in rodents. This led to the major medical breakthrough in the eradication of rickets and the discovery of vitamin D between the years of 1919 to 1924.	Steenbock, 1924. Hess and Weinstock, 1924.
The discovery of vitamin D came with its own challenges because an outbreak of hypercalcaemia in 1950 was thought to be due to excessive vitamin D concentrations in foodstuffs. Authorities banned fortification of food or personal use of products with vitamin D in Great Britain. The ban spread to other European countries, except for margarine and cereal fortified with vitamin D. However, Pober, (101) has suggested that the hypercalcaemia reported in the UK might have been due to hypersensitivity to vitamin D as a result of a condition called Williams Beuner Syndrome (WBS).	Lightwood et al., 1956. Tshibangu et al., 1975.

Milestones in Vitamin D research

Development of assays to measure 1,25 $(OH)_2D$ and 25(OH)D.	**1,25 $(OH)_2D$ Assay** Competitive binding assay, Eisman et al., 1976. Isotope dilution-mass fragmentography assay, Björkhem et al., 1979. The cytoreceptor assay, Manolagas et al., 1983. **25(OH)D Assay** Competitive protein binding assays (CPBA), Haddad and Chyu, 1971. HPLC, Eisman et al., 1977, Gilbertson and Stryd, 1977. Radioimmunoassay (RIA), Hollis and Napoli, 1985. LC-MS/MS, Higashi et al., 2002, Eyles et al., 2009.
Controversies in Vitamin D definitions by various scientific institutions.	IOM, Ross et al., 2011a. US Endocrine society, Holick et al., 2011. UK, Scientific Advisory Committee on Nutrition, 2016.
The vitamin D external quality assessment scheme (DEQAS) has been monitoring 25(OH)D assays performance since 1989.	Hollis, 2008.
Call for vitamin D standardization.	Binkley et al., 2004, Sempos et al., 2012.

The discovery of the VDR in rat and human skeletal cells. This led to increased attention from muscle physiologists on the potential role of vitamin D in regulation of protein synthesis and muscle function.	Simpson et al., 1985. Bischoff- Ferrari et al., 2004. Hamilton, 2010.

2.5 Factors influencing vitamin D status

Vitamin D status is influenced by a multitude of factors, with major factors categorized into environmental or external and personal factors. Environmental factors include latitude and season, air pollution and high-density living. Personal factors include age, melanin pigmentation of the skin, religious and cultural practices. Body composition and genetics influence 25(OH)D levels as well.

2.5.1 Latitude and season

Latitude and season are both responsible for the quantity and quality of solar radiation reaching the earth's surface (Webb et al., 1988). The ability to synthesize previtamin D_3 is affected by latitude, rotation of the earth about the sun (seasonally) and its own axis during day and night (Harinarayan et al., 2013). Individuals living progressively further from the equator are exposed to lesser amounts of UV light due to the greater solar zenith angle. The solar zenith angle (SZA) is the angle between the local vertical (zenith) and the position of the sun at any given moment. The SZA is constantly changing due to the earth's daily rotation on its axis and its orbit around the sun (Kimlin, 2008). Figure (page 17) shows that at a large SZA the radiation traverses a longer path-length through the atmosphere, which can greatly attenuate shorter wavelengths. Furthermore, the attenuated power is spread over a larger area of the surface. Serum 25(OH)D levels may vary depending on season. As the seasons change, there is either an increase or decrease in SZA and more or less UVB photons are absorbed by the stratospheric zone.

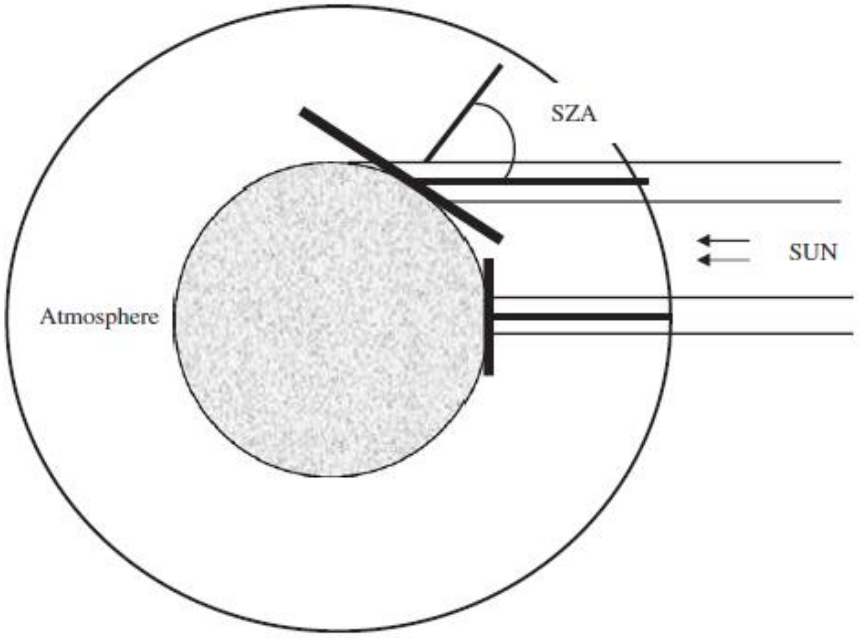


Figure 2.3: Solar zenith angle (SZA), the angle between the local vertical and the position of the sun in the sky. Source: Webb, 2006.

2.5.2 Seasonal variations

The exposure of the skin to ultraviolet light is affected by seasonal variations, which may have an impact on subjects of all ages. The past clinical experiences and observations of children with rickets in studies reported in the 1970s indicated that the disease was common in spring but seldom occurred in autumn, suggesting that ultraviolet light in summer is a vital source for cutaneous vitamin D production (Stamp and Round, 1974, Mclaughlin et al., 1974). Another important finding was the development of biochemical assays for vitamin D metabolites which led to examination of the relative importance of diet and endogenous skin synthesis (Haddad and Hahn, 1973) and to the initial findings of seasonal variation in plasma 25(OH)D levels in cross-sectional and longitudinal studies (Stamp and Round, 1974, Mclaughlin et al., 1974). Studies have reported seasonal 25(OH)D variations in different ethnic groups (Livesey et al., 2007, Shoben et al., 2011, Heidari and Haji Mirghassemi, 2012) but not all studies agree with this finding (Meier et al., 1991, Haarburger et al., 2009). The importance of skin colour in seasonal variation has been reported: a study of dark-skinned Pacific Islanders and individuals of European ancestry or light-skinned Asians reported that 25(OH)D levels were twice as high in lighter-skinned subjects than in the darker-skinned group across all seasons (Scragg et al., 2004).

In another study in the US (Boston) healthy adolescents were reported to have lower 25(OH)D levels (24%, ≤ 37.5 nmol/L) during winter as compared to summer (Gordon et al., 2004). Low 25(OH)D levels were attributed to low levels of ultraviolet light exposure during winter in Boston, during which period other researchers have suggested that children from both northern or southern latitudes should be advised to receive vitamin D supplementation (Harkness and Cromer, 2003, Weaver and Fleet, 2004). Hypovitaminosis D is a known problem across the globe (Lips, 2007, Bassil et al., 2013) and seasonal variations in 25(OH)D in children are known to affect bone turnover and in turn, potentially bone accrual and peak bone mass (Rajakumar et al., 2013).

South African studies on seasonal variation are a handful (Pettifor et al., 1996, Haarburger et al., 2009, Martineau et al., 2011b). An interesting variation was reported by Pettifor et al., 1996 *in vitro*, where vials containing 7-DHC were exposed to sunlight one day per month from 08:00 am and 17:00 pm, in two cities in South Africa (SA) (Johannesburg and Cape Town). There was marked seasonal variation in vitamin D_3 observed in Cape Town, with very little being formed during the winter months of April through September. In Johannesburg, *in vitro* formation changed slightly throughout the year, and was similar to that found in Cape Town during the summer season. A retrospective study in Cape Town also found no significant difference of 25(OH)D levels (49 vs 47 nmol/L, $p > 0.05$) in seasonal variation in the summer and winter season (Haarburger et al., 2009). However, the study was biased because it was dependent on 25(OH)D estimations being requested by physicians suspected for vitamin D deficiency. There were fewer sample requests during summer in comparison to winter, suggesting that there might well have been a seasonal variation in the incidence of vitamin D deficiency. The previously documented seasonal variation in serum 25-hydroxyvitamin D recorded in patients in Johannesburg might be as a probable consequence of the increased clothing worn and the decreased time spent out of doors during winter, rather than decreased ultraviolet radiation reaching the earth (Pettifor et al., 1978b). Other studies in SA have assessed vitamin D status but they did not report on seasonal variations (Kruger et al., 2011, Lategan et al., 2016).

2.5.3 Duration of time spent out in the sun and sunscreen

Studies have reported that exposure of the arms and head to sunlight for approximately 15 minutes for 3 days per week may result in sufficient vitamin D to meet the daily requirements

(Chel et al., 1998, Luxwolda et al., 2012, Lategan et al., 2016). However, the messages from studies in terms of time spent outdoors and sunscreen applications are contradictory at present. The benefits of spending time outdoors are associated with sunlight exposure on the skin resulting in the cutaneous vitamin D production (Luxwolda et al., 2012, Lategan et al., 2016). However, skin exposure to sunlight has been associated with skin cancer (Lombardi et al., 2013). Hence, people are advised to use sunscreen with sun protection (SP) factor of 15 or greater in order to avoid skin cancer (Lautenschlager et al., 2007). The issue is that applying sunscreen has the potential of preventing the cutaneous vitamin D production which may result in low vitamin D status (Narayanan et al., 2010). But vitamin D production may increase when a thinner sunscreen layer (< 2 mg cm-2) is applied (Faurschou et al., 2012). There is a need to be a balance between the advocates for preventing skin cancer and those wishing to prevent hypovitaminosis D in order to achieve 'sufficient' vitamin D without harming the skin.

2.5.4 Air pollution and high density living

Exposure to chronic air pollution generated as a consequence of urban activities such as automobile exhaust fumes, heating of buildings and manufacturing in industrial plants constitutes an environmental health risk (Barrea et al., 2017). One of the global health risks of air pollution is the reduction of UVB radiation reaching the human skin, which is suggested to be a major cause of vitamin D deficiency in India (Agarwal et al., 2002) and elsewhere (Bailey et al., 2012, Wacker and Holick, 2013). It has been reported that people living in urban areas, especially in areas with high levels of ambient air pollution, tend to be less engaged in outdoor activities and have a higher prevalence of hypovitaminosis D (Bailey et al., 2012).

High-density living is also thought to contribute to the prevalence of vitamin D deficiency. High rise buildings in close proximity to each other prevent UV rays reaching pavements and recreation areas, thereby making endogenous production of vitamin D a challenge (Wacker and Holick, 2013).

2.5.5 Age

Age is considered to be a factor in diminishing the cutaneous production of vitamin D. In elderly subjects in Britain 25(OH)D levels were lower in the non-housebound elderly, even during the summer months when compared to younger British subjects (Lester et al., 1977). Hence, they

suggested that aging might decrease the efficiency of vitamin D_3 formation in the skin. Furthermore, MacLaughlin and Holick, 1985 showed in a study of Caucasian human skin samples from subjects aged 8 to 92 years, that the total amount of previtamin D_3 that was produced in the epidermis and dermis of the younger subjects was at least two times greater than that of the elderly subjects. It has also been suggested that hypovitaminosis D in elderly subjects is related to decreased vitamin D receptor quality (Lau and Baylink, 1999), expression (Bischoff-Ferrari et al., 2004) and age-related decline in kidney function (Vieth et al., 2003).

Low vitamin D status in older people in England continues to be a public health problem. The prevalence of vitamin D deficiency (25(OH)D <25 nmol/L) in people aged ≥ 65 years in 2005 was 13% in women and 8% in men and the risk of hypovitaminosis D was found to increase with age (Hirani et al., 2009). Hypovitaminosis D in the elderly has a significant impact on physical, psychological, and monetary status (buying of vitamin D supplements and transport to the nearest healthcare centre) and becomes a burden to society (Tuck and Francis, 2002). According to the Scientific Advisory Committee on Nutrition, 2016, vitamin D deficiency in the elderly may be due to inadequate exposure to sunlight and chronic conditions associated with old age like compromised kidney and liver function.

2.5.6 Melanin pigmentation of the skin

Cutaneous production of previtamin D_3 occurs when the skin is exposed to solar radiation at a wavelength of approximately 290-315 nm (Holick., 1981). Vitamin D status in populations is strongly associated with the degree of skin pigmentation. People with darker skin colour from the African continent (Daniels et al., 1997, Kruger et al., 2011), Asian subcontinent (Marwaha et al., 2005, Marwaha et al., 2011) and from the USA (African Americans) and also Africans in the diaspora have lower vitamin D status compared to people with lighter skin colour (Harris, 2006, Weng et al., 2007, Cosman et al., 2007, Looker et al., 2008). However, not all studies show similar findings (Luxwolda et al., 2012, Lategan et al., 2016). Increased melanin pigmentation is regarded as the major cause for the lower 25(OH)D levels because it influences the penetration of sunlight or UV radiation into the skin. Skin pigmentation is important in regulating the production of vitamin D_3 especially in circumstances of low levels of irradiation because melanin absorbs UV photons in competition with 7-dehydrocholesterol.

Studies conducted in migrants who have relocated to Europe and Australia have found those with dark skin colour have low vitamin D status (Lo et al., 1986, Eggemoen et al., 2013, Brock et al., 2013). However, people with dark skin colour have the ability to produce similar vitamin D concentrations to lighter skinned individuals when exposed to prolonged solar radiation (Brock et al., 2013).

2.5.7 Religious and cultural practices

The extent of skin coverage by clothing is an important factor influencing the amount of UVB radiation reaching the skin and thus the amount of vitamin D_3 formed cutaneously. Certain types of attire such as the wearing of a niqab or hijab, as dictated by religion or culture in some communities, can play a crucial role in preventing sunlight exposure to the skin (Batieha et al., 2011).

In some cultures or religious practices, it is the norm that women and girls cover their bodies with clothes most of the time, thereby not exposing their skin to the ultraviolet rays from sunlight (Al-Saleh et al., 2013, Alsuwaida et al., 2013). Countries such as India (Agarwal et al., 2002, Marwaha et al., 2011), Iraq (Maalouf et al., 2007), Lebanon (Nabulsi et al., 2008), Bangladesh (Roth et al., 2010), Morocco (El et al., 2012), Kuwait (Al-Mutairi et al., 2012), Pakistan (Khan et al., 2012b), Jordan (Nichols et al., 2012), Iran (Andıran et al., 2012), Saudi Arabia (Alsuwaida et al., 2013), United Arab Emirates (UAE) (Muhairi et al., 2013), Syria (Sayed-Hassan et al., 2014) have enough sunlight throughout the year but their populations experience low vitamin D status due to their cultural way of living.

2.5.8 Body composition

Individuals with increased body mass index (BMI) and body fat content have lower 25(OH)D than their lean peers (Ghergherechi et al., 2012). Vitamin D is fat-soluble and thus absorbed in the adipose tissue cells (Wortsman et al., 2000, Arunabh et al., 2003), which is thought to reduce available circulating vitamin D and thus lower 25(OH)D levels in obese and overweight people. Therefore, an increase in body weight due to fat deposition or obesity influences vitamin D status negatively, which may result in hypovitaminosis D (Palacios et al., 2012). However, the exact mechanism responsible for this effect is not clear; it has been suggested that obese people have a tendency of avoiding sunlight, have more sedentary lifestyles, cover their bodies more completely with clothes (Compston et al., 1981, Arunabh et al., 2003, Abrahamsen, 2017) and

have fewer outdoor activities compared to lean individuals (Harel et al., 2011), resulting in less formation of cutaneous vitamin D_3. The metabolic clearance of vitamin D may also be increased in obese subjects due to its lipid-soluble nature, resulting in decreased bioactivity of vitamin D. Based on these findings, authors have suggested that vitamin D supplementation especially in overweight and obese young children might be beneficial for bone status and other health conditions (Zittermann et al., 2009, George et al., 2012).

Population studies of some ethnic groups have found that the combination of dark skin and obesity increases the chance of developing hypovitaminosis D. Researchers have also acknowledged obesity as a major public health problem in the global arena (Duncan et al., 2004, Kelly et al., 2008, Abolfotouh et al., 2012, Malaza et al., 2012, Kumar and Kaufman, 2018, Tarp et al., 2018). In both children and adults, obesity is associated with secondary hyperparathyroidism (Buyukinan et al., 2012), insulin resistance (Kelly et al., 2011, Buyukinan et al., 2012), type 1 diabetes (Giulietti et al., 2004), type 2 diabetes (Buyukinan et al., 2012), hypertension (Forman et al., 2007), dyslipidemia (Baker et al., 2012), and cardiovascular diseases (Jorde and Grimnes, 2012). Obesity is a condition that is frequently associated with vitamin D deficiency (Buyukinan et al., 2012) and some studies have shown obesity to be inversely correlated with 25(OH)D levels (Compston et al., 1981, Nam et al., 2012). An inverse relationship between BMI and 25(OH)D levels has not been found consistently across all ethnic groups. A study by Nesby-O'Dell et al., 2002 found that vitamin D deficiency was significantly associated with a BMI of at least 30 kg/m^2 in white women, but not in African American women. In that particular study, the researchers speculated that differences in fat distribution among ethnic groups may be a factor. However, studies in children have found low levels of 25(OH)D in obese children to be in line with most studies in adults (Bell et al., 1985, Buffington et al., 1993, Wortsman et al., 2000, Parikh et al., 2004, Reinehr et al., 2007).

A possible contributory factor to the high prevalence of obesity in African Americans and black Africans may be the low calcium content of diets (Zemel, 2002). It is likely that diets low in calcium are also rich in fat and carbohydrate (Zemel, 2001, Jacqmain et al., 2003). However, the role of low calcium diets in the pathogenesis of obesity is still unclear (Lappe et al., 2017, Marabujo et al., 2018). Using the National Health and Nutrition Examination Survey (NHANES) data, researchers have concluded that a low-calcium diet favoured an increase in adipose tissue

and that the opposite is true for calcium-rich diets, thus prompting the suggestion that calcium has an anti-obesity effect (Zemel, 2002). Theoretically, an increase in dietary calcium would suppress 1,25$(OH)_2$D and PTH, thereby decreasing adipocyte intracellular calcium and lipid storage (Zemel et al., 2000). However, some researchers are not yet convinced about the role of calcium in obesity (Heaney and Rafferty, 2009). Recently a Korean National Health and Nutrition Examination Survey (KNHANES) in adults, conducted using a food frequency questionnaire and a one-day 24-hour recall, reported that consumption of dairy products was protective against obesity (Odds ratio (OR) = 0.63, 95% confidence interval (CI) = 0.68 – 0.96). The authors have emphasized that longitudinal studies are still warranted to replicate their findings (Lee et al., 2014).

2.5.9 Genetic factors

Studies on genetic drivers of vitamin D status have started to emerge that have shown that genetic factors also play a role in controlling 25(OH)D levels in populations (Shea et al., 2009, Signorello et al., 2011). Common genetic variants of several genes controlling pathways that synthesize, transport and degrade vitamin D may play a role in individual (and potentially population) differences in vitamin D status. Presently, vitamin D pathway candidate genes have been considered together with single nucleotide polymorphisms (SNPs). These include vitamin D receptor (VDR), 1-α-hydroxylase (CYP27B1), 25-hydroxylase (CYP2R1), 24-hydroxylase (CYP24A1), vitamin D binding protein (GC, DBP) and vitamin D_3 25-hydroxylase (CYP27A1) genes. Genetic polymorphisms including variants near genes involved in cholesterol synthesis (DHCR7 - 7-dehydrocholesterol reductase), hydroxylation (CYP2R1) and (CYP24A1) and vitamin D transport (GC) may identify individuals at high risk of vitamin D deficiency especially in ethnic groups with darker skin colour. A study of 94 single nucleotide polymorphisms (SNPs) in 5 vitamin D pathway genes (GC, VDR, CYP2R1, CYP24A1, CYP27B1) and serum 25(OH)D levels among African Americans and Caucasians showed statistical associations for 3 SNPs (rs2298849 and rs2282679 in the GC gene, and rs10877012 in the CYP27B1 gene), in African Americans (Signorello et al., 2011). Recently, Jiang et al., 2018 conducted a study in 79366 individuals of European ancestry which confirmed the role of common genetic variants in the regulation of 25(OH)D concentrations. Furthermore, their SNP-heritability results suggested that 25(OH)D has a modest overall heritability due to common genome-wide SNPs of 7.5% and that an appreciable proportion (2.84 out of 7.5 i.e 38%) of this total could be explained by known

genetic regions identified through Genome-Wide Association Studies (GWAS). This was in line with the previously published findings, wherein the proportion of variance was explained by 8.9%, which was done by employing a linear mixed model fitting the additive genetic matrix created from all genotyped and imputed SNPs (Hiraki et al., 2013).

2.6 Challenges in vitamin D status definition and cut-off range in children

The definitions of vitamin D status in terms of its deficiency, insufficiency and sufficiency or "optimal" levels are still subject to debate by researchers. At present, there is no consensus on the exact definition of the optimal vitamin D status in children, adults and the elderly for bone health and other diseases. Great variation exists between reports when defining deficiency, insufficiency and adequate levels of vitamin D (Sadat-Ali et al., 2009, Balasubramanian et al., 2013). The lack of consensus makes interpreting reports a major challenge. But at present the vitamin D definition debate will still continue until there is consensus among researchers (Holick et al., 2011, Rosen et al., 2012, Fraser and Milan, 2013). The difference in reporting of vitamin D cut-off ranges may be the attributes of lack of consensus in vitamin D status definition. With respect to the vitamin D status, this definition of normality is not appropriate as 25(OH)D values may vary by population (skin colour and lifestyle), seasons of the year and latitude (low and high) and the type of laboratory assay (Table 2.3, page 26) which has been shown by the majority of published papers in different parts of the world. Due to the lack of consensus in vitamin D status definition, researchers have continued using different vitamin D cut-offs as published from scientific institutions such as IOM (Ross et al., 2011a, Rosen et al., 2012), US endocrine society (Holick et al., 2011) and more (Pludowski et al., 2013, Scientific Advisory Committee on Nutrition, 2016), based on physiology and logic.

The cut-off ranges of vitamin D status in children have been reported in various regions of the world, but with lack of consistency (Docio et al., 1998, Zittermann, 2003, Weaver and Fleet, 2004, Kumar et al., 2009). A study in prepubertal children has reported disturbances in calcium homeostasis when 25-hydroxyvitamin D levels were between 30 and 50 nmol/L and it was also demonstrated that concentrations of 50 nmol/L and above stabilized PTH (Docio et al., 1998). However, in a similar study in children, it was reported that PTH increases when 25-hydroxyvitamin D is below 30 – 40 nmol/L. Hence, it is suggested that further studies are needed to verify whether the set levels of serum 25-hydroxyvitamin D have been accurately/perfectly

established in children and adolescents (Docio et al., 1998, Outila et al., 2001). According to researchers the "normal" range for 25(OH)D levels is broad (12.5 – 120 nmol/L) and the lower limit can vary across populations (Zittermann, 2003, Weaver and Fleet, 2004). In a US study (NHANES 2001 – 2004) by Kumar et al., 2009, 9% of children had 25(OH)D levels of < 37.5 nmol/L and 61% of children and adolescents had levels between 37.5 – 72.5 nmol/L representing approximately 51 million US children. After adjustments for confounders in multiple regression, children with vitamin D deficiency had lower serum calcium levels and HDL cholesterol levels and higher systolic blood pressure than those with 25(OH)D levels of ≥ 75 nmol/L. Children and adolescents with 25(OH)D of between 37.5 – 72.5 nmol/L had lower levels of total cholesterol and HDL cholesterol and higher diastolic blood pressure and elevated PTH than their counterparts with 25(OH)D of ≥ 75 nmol/L. In the United Kingdom (UK), a serum 25(OH)D concentration of 25 nmol/L is currently used as a threshold cut-off for defining the lower limit of adequacy, which is based on evidence indicating that the risk of bone disease (rickets and osteomalacia) is related to 25(OH)D concentrations below 25 nmol/L (Prentice, 2016). Table 2.3 (page 26) shows the variation in serum 25(OH)D used by researchers to define vitamin D status.

Table 2.3: A snapshot of various cut-off points used by researchers in children and adults to define vitamin D status based on laboratory methods

Researcher	Country	Latitude	Population	No of Participants	Age	Lower cut–off used to define vitamin D status (25(OH)D (nmol/L))	Method
			Children				
Guillemant et al., 1999	France	49 ^{o}N	Male adolescents	175	13-17 years	Deficiency < 25 Insufficient < 50 Sufficiency ≥ 50	CPBA
Fuleihan et al., 2001	Lebanon	33.5 ^{o}N	Healthy school children	346	10-16 years	Deficiency < 25 Insufficient 25 - 50 Sufficiency ≥ 50	RIA
Outila et al., 2001	Finland	60 ^{o}N	Healthy female adolescents	178	14–16 years	Deficiency ≤ 25 Insufficient 25.1 - 40 Sufficiency > 40	RIA
Lehtonen-Veromaa et al., 2002	Finland	60 ^{o}N	Girls	191	12.9 years	Deficiency < 20 Insufficient 20.1 – 37.4 Sufficiency > 37.5	RIA
Cheng et al., 2003	Finland	60 ^{o}N	Girls	193	10-12 years	Deficiency ≤25 Insufficient 26 - 40 Sufficiency > 40	RIA
Gordon et al., 2004	US	40 ^{o}N	Healthy Adolescents	307	14.7 years	Deficiency ≤ 16 Insufficient 16.1 - 50	CBA

						Sufficiency > 50	
Cashman et al., 2008	Northern Ireland	55°N	Adolescents	1015	12-y-Boys 12-y-Girls 15-y-Boys 15-y-Girls	Deficiency < 25 Insufficient - 25.1 - 50 Sufficiency - > 50	ELISA
Cashman et al., 2011	Denmark Finland	55°N 60 °N	Danish girls Finnish girls	47 97	11.3 years	Deficiency - < 25 Insufficient 25.1 – 49.9 Sufficiency ≥ 50	HPLC
Mansour and Alhadidi, 2012	Saudi Arabia	24 °N	Healthy children	510	4 -15 years	Deficiency < 17.5 Insufficient 17.6 - 50 Sufficiency > 50	CLIA
Marrone et al., 2012	Italy	42 °N	Children	93	2-220 months	Severe deficiency < 12.5 Deficiency < 37.5 Insufficient 37.5 – 50 Sufficient > 50	CLIA
			Adults				
Thomas et al., 1998	US	40 °N	Medical in patients	290	43–81 years	Deficiency 20 Insufficient 20.1 – 37.5 Sufficiency > 37	CPB
Harris et al., 2000	US	40 °N	Osteoporotic	246	67-83 years	Deficiency < 25 Insufficient 25.1 - 50 Sufficiency > 50	CPB
Kauppinen-Mäkelin et al., 2001	Finland	60 °N	In and Out Patients	205	58-65 years	Deficiency 37.5 Insufficient 37.1 - 50	RIA

						Sufficiency	> 50	
Gómez-Alonso et al., 2003	Spain	43^{o}N	Osteoporotic	268	54-89 years	Deficiency Insufficient Sufficiency	< 25 25 - 45 > 45	CPB

The table shows that researchers from across the globe have used different cut-off points in defining vitamin D status. This reflects the challenge encountered by the researchers in defining vitamin D status based on biological abnormalities.

2.7 25(OH)D assays employed in various laboratories

The measurement of 25(OH)D has been a great challenge to researchers through the years, because of significantly varying results generated by many differing methods, which are briefly described below.

2.7.1 Competitive protein binding (CPB) assays

This method was the first assay developed to measure 25(OH)D at the beginning of the 1970s. It used a preliminary solvent extraction of samples, and a chromatographic purification result which removed vitamin D binding protein (DBP) (Haddad and Chyu, 1971). The assay used an extract which contained an unknown, unlabelled 25(OH)D, which is incubated with a known concentration of radioactively labelled 25(OH)D_3. The labelled and unlabelled compound competed for an added limited amount of binding protein. CPB assays utilized rachitic rat, chicken, or human-derived binding protein and there is a correction of procedural losses during extraction and purification processes. The assays had a short incubation time of one hour and had a great advantage of measuring across a wide range of concentrations. Belsey et al., 1971 developed a CPB assay utilizes a specific vitamin D binding protein from rat serum, and the researcher claimed that they do not require chromatographic extraction as a preliminary step. It was considered to be sensitive and specific enough for practical use in measuring the plasma concentration of 25(OH)D in animals and man.

The disadvantages of these assays were that they cross-reacted with several 25(OH)D metabolites (3-epi-25-OHD_3, 24,25-dihydroxyvitamin D (24,25$(OH)_2$D), and the binding protein lacked stability in preparations (Dorantes et al., 1978). However, attempts have been made to automate a CPB-based assay but it has proved to be difficult due to its assay variability, poor detection limit, low cross-reactivity with 25(OH)D_2, poor precision and overall unacceptable performance (Leventis et al., 2005, Glendenning et al., 2006).

2.7.2 Immunoassays

In the 1980s the first radioimmunoassay utilized radioactive ^{3}H-labelled 25(OH)D as a tracer. This was subsequently changed to a ^{125}I-labelled assay with higher throughput and improved outcomes (Hollis and Napoli, 1985, Hollis et al., 1993). These assays have now given way to a

chemiluminescent detection based system, which is automated and free from radioactive exposure (Heijboer et al., 2012).

Several manufacturers have produced 25(OH)D immunoassays in which solvent extraction and chromatographic separation have been replaced by agents that displace 25(OH)D from DBP with ease. This approach has prompted the automation of these assays but some data suggest that some immunoassays employing these techniques may be affected by variations in DBP concentrations. This is due to variable displacement of 25(OH)D from DBP and in specific subjects may be due to increased affinity of 25(OH)D for certain variants of DBP leading to marked variations in outcomes (Heijboer et al., 2012). An area of concern in relation to immunoassays is the variability that exists in the detection of $25(OH)D_{2,}$ because of different binding affinities to DBP with $25(OH)D_3$ exhibiting a stronger affinity than $25(OH)D_2$ (Jones, 1978). But, some manufacturers claim to peers have 100% cross-reactivity with exogenously added $25(OH)D_2$ and $25(OH)D_3$ and therefore equipotent for the measurement of the two metabolites (Glendenning et al., 2006, Binkley et al., 2010).

2.7.3 HPLC and LC-MS/MS

The use of UV detection-based HPLC assays for 25(OH)D was first reported in 1977 (Eisman et al., 1977, Gilbertson and Stryd, 1977). The method is however considered a cumbersome and laborious technique which involves chloroform-methanol extraction followed by chromatography on sephadex/silica gel columns, followed by HPLC with UV detection (Fraser and Milan, 2013). Therefore, this technique cannot be routinely used in a normal clinical laboratory requiring a fast turnaround time.

The use of LC-MS/MS has gained momentum and has become the method of choice in measuring steroids such as 25(OH)D. This is because it requires small sample volumes, has fast analysis times and improved specificity (Keevil, 2013). However, LC-MS/MS has a number of shortcomings, because it requires the removal of protein and other interfering substances from the sample before analysis. In most instances, the protein molecules block the instrument leading to failure in achieving the required concentration of the analyte (Taylor, 2005).

2.8 The need for standardization of 25(OH)D assays

The varied performance of 25(OH)D assays in various laboratories has resulted in the establishment of the International External Quality Assessment Scheme for vitamin D metabolites (DEQAS) over the past three decades (Mayer and Schmidt-Gayk, 1984, Carter and Short, 1988). This establishment was aimed at improving the accuracy and precision of the 25(OH)D assays. DEQAS was formed to allow scientists to assess the reproducibility of their assay and to compare their assay results to assays using the same technique and with different assay techniques (Carter et al., 2004). Researchers have called for vitamin D standardization because assay variations were found to confound the diagnosis of hypovitaminosis D. This is because whether an individual is found to have low or normal vitamin D status was a function of the laboratory being used (Binkley et al., 2004). A potential confounder between assays is the interference of other circulating vitamin D metabolites (Carter et al., 2016). Due to regular monitoring of 25(OH)D between laboratories across the world on a quarterly basis by DEQAS, it is acknowledged that 25(OH)D assays have improved, with better agreement between laboratories and methods (Carter et al., 2010). Since the inception of DEQAS (Carter and Short, 1988), vitamin D assays have come full circle from rigorous extraction, chromatographic methods to less rigorous methods to the modern equivalent of extraction/chromatographic assays conducted in specialist laboratories (Carter et al., 2018). But researchers have acknowledged that 25(OH)D remains a "difficult" analyte to measure due to its hydrophobic nature, its existence in several different molecular forms and its tight binding to DBP (Carter, 2012).

For quite some time, the absence of a recognized international reference standard for 25(OH)D_2 and 25(OH)D_3 in assays has meant that most vitamin D researchers have produced their own standard and calibrator material for HPLC and LC-MS/MS methods. The initial idea was to obtain the purest form of 25(OH)D_2 or 25(OH)D_3 available commercially and dissolve these in sufficient quantity in ethanol. This was to enable spectrophotometric estimation of absorbance at both 265 nm (maximum absorption) and 228 nm (minimum absorption) to evaluate for purity. The ultimate step was to use the appropriate molar absorption coefficient (MAC) to calculate an accurate concentration of 25(OH)D_2 and 25(OH)D_3 in a sample. However, this approach was not error-free; its problems were associated with the purity and source of 25(OH)D and the need to dissolve 25(OH)D in an organic solvent. The diluent/matrix used to make subsequent standard dilutions had resulted in the presence of DBP of different species with varying affinities for

$25(OH)D_2$ and $25(OH)D_3$, and the marked variability in the MACs used (Fraser et al., 2013). The standardization of 25(OH)D is still work in progress, but the elimination of matrix effects associated with DBP is a challenge, and will require the continual vigilance of analysts and clinicians and ongoing dialogue with manufacturers (Carter, 2012, Carter et al., 2018).

The researchers have recognized the advantages that are to be achieved from creating an international vitamin D status database. Hence the US office of Dietary Supplements has organized a project, the Vitamin D Standardization Program, (VDSP) with the aim of standardizing measurements conducted in national health surveys to those of the Reference Measurement Procedure (RMPs) developed by NIST (National Institute of Standards and Technology) and the University of Ghent (Coates, 2011, Sempos et al., 2012). This is because epidemiological studies can provide information on 25(OH)D normative data but differences in methods used in national surveys have limited the extent to which the results can be pooled (Carter, 2012). As a result, laboratory standardization in vitamin D research is an essential element in developing consensus regarding the 25(OH)D to define vitamin D status (Bouillon, 2017, Binkley et al., 2017, Herrmann et al., 2017). In order to achieve vitamin D standardization researchers (Sempos et al., 2020), assigned to VDSP have established criteria for selecting an assay, which is as follows:

- ❖ Fit for use – The assay for the required test must perform appropriately and provide standardised measurements in the patient/study populations in the conditions for which it will be used.
- ❖.The assay must be certified by the centre for disease control and prevention (CDC) vitamin D standardization certification program as being standardised and having an appropriate measurement range or be a documented standardised laboratory-developed HPLC or LC-MS/MS assay with an appropriate measurement range.
- ❖The assay is expected to have appropriated level of precision and accuracy.
- ❖ The assay is required to meet VDSP assay criteria wherein the total CV is $\leq 10\%$ and mean bias with the range of -5 to +5%.

2.9 Vitamin D status across the continents of the world

Vitamin D status has been documented widely across different communities (Prentice, 2008, Mithal et al., 2009, Van Schoor and Lips, 2011). Difficulties in determining the prevalence of vitamin D deficiency among different communities is the result of several factors such as:

1. Defining the cut-off points used to define vitamin D deficiency.
2. The season of the year in which the study was conducted.
3. The assay used to determine the 25(OH)D concentrations.

2.9.1 South Africa

Several studies on vitamin D status have been conducted in South Africa on vitamin D status dating back to 1978 (Pettifor et al., 1978a, Pettifor et al., 1978b, Daniels et al., 1997, Kruger et al., 2011, Martineau et al., 2011b, Velaphi, 2017). In Johannesburg, elderly South African patients with femoral neck fractures were observed to have low (25(OH)D, 44.3 nmol/L) as compared to children living in the same area (25(OH)D, 76.8 nmol/L) (Pettifor et al., 1978a). In another study conducted in infants and children of mixed ancestry (N =285, aged 1 to 17 years), the 25(OH)D was lower in older children aged around 8 years (85.5 to 56.8 nmol/L) and it was suggested that these children spent less time outdoors because of engagement with school activities (Pettifor et al., 1978b). In a group of nurses, black nurses had significantly lower 25(OH)D (47.5 versus 64.5 nmol/L, p = 0.0001) and higher $1,25(OH)_2D$ (35.2 versus 26.1 pg/ml, p = 0.0001) compared to their white peers (Daniels et al., 1997). These findings are similar to those reported from the US, where African Americans have lower 25(OH)D and higher $1,25(OH)_2D$ than white Americans (Harris, 2006). In a study conducted in Mangaung (Free State Province) on urban black communities, the mean 25(OH)D of 96 ± 11.2 nmol/L was found to be 'adequate' (96%) for vitamin D status (> 50 nmol/l), with only 4% having 25(OH)D levels between 30 - 50 nmol/L (Lategan et al., 2016). It was suggested by the authors that the good 25(OH)D levels were due to latitude, a sunny climate, and sufficient sun exposure.

With the HIV pandemic being on the rise in early 2000 and researchers being aware of the non-calcaemic vitamin D functions, there was an interest in associating vitamin D with HIV, TB and other disease conditions (Djennane et al., 2014b). In a study by Martineau et al. 2011b in Cape Town, 67.0% of young adults (aged 19.7 – 38.2 years) were vitamin D deficient (< 50 nmol/L).

Vitamin D deficiency was associated with susceptible to active TB, both in the presence and absence of HIV infection.

2.9.2 Summary of vitamin D status in other continents

2.9.2.1 Africa

Several studies assessing vitamin D status have been conducted in various parts of the African continent and also in the African diaspora. From North Africa, Djennane et al., 2014b study of Algerian children and adolescents (5 - 15 years) found that vitamin D insufficiency (25(OH)D < 50 nmol/L) to be present in 52% of the studied population despite a sunny environment. In Morocco, studies in healthy children are still lacking. Bouaddi et al., 2014 in a study of Moroccan children with juvenile idiopathic arthritis have found hypovitaminosis D (25(OH)D of < 50 nmol/L) to be 75%. In addition, a study in adults has also reported a hypovitaminosis D of 91% (25(OH)D < 75 nmol/L) (Allali et al., 2009).

In West Africa (Nigeria), children aged between 3 and 36 months were found to have calcium insufficiency rather than vitamin D deficiency, the former most likely contributing to the etiology of rickets in this age group. The 25(OH)D concentrations were considered to be 'normal', supporting the contention that rickets in this population of children, which is due to vitamin D deficiency, is an uncommon phenomenon (Pfitzner et al., 1998). Thacher et al., 2012 also reported similar findings in other Nigerian children.

In Tanzania, (East Africa), a cross-sectional study of vitamin D status among 191 HIV-exposed uninfected 6-month-old infants enrolled in a randomized, double-blind, placebo-controlled trial was conducted. A third of the subjects were classified as vitamin D deficient (< 50 nmol/L), half were vitamin D insufficient (50 – 75 nmol/L, 48.7%) and less than 20% as vitamin D sufficient (≥ 75 nmol/L). Independent risk factors for vitamin D deficiency were sampling during the rainy season and infant wasting (Rwebembera et al., 2013). Another study in Tanzania, conducted in infants at 6 weeks and again at 6 months of age, born to HIV-uninfected mothers, found the prevalence of vitamin D deficiency (<50 nmol/L) to decline from approximately 76% at 6 weeks of age to 21% at 6 months. However, there was no association of vitamin D status at 6 weeks or 6 months of age with mean length for age Z-score (LAZ) or weight for length Z-score (WLZ)

trajectory, nor with the incidence of stunting, wasting, or being underweight (Sudfeld et al., 2017).

2.9.2.2 North America

Data from the United States had showed the prevalence of hypovitaminosis D to be significantly higher in African Americans as compared to their Caucasian counterparts, irrespective of age (Perry et al., 1993, Nesby-O'Dell et al., 2002, Rucker et al., 2002, Harkness and Cromer, 2005a, Mansbach et al., 2009, Langlois et al., 2010). In these studies, there was no uniformity in categorizing vitamin D status in terms of deficiency, insufficiency and sufficiency and there were no consistencies in reporting of units for 25(OH)D; some researchers used nanomole per litre (nmol/L), while others reported the results in nanogram per millilitre (ng/ml). Different assay methodologies in testing for 25(OH)D were also used, hence making comparisons between laboratories a challenge.

Despite some foodstuffs especially milk being fortified with vitamin D, there are still reports of hypovitaminosis D among the different ethnic groups in the US (Harkness and Cromer, 2005a). In a children's study conducted in Philadelphia, Pennsylvania area (Latitude 40^0 N), the prevalence of hypovitaminosis D (< 75 nmol/L) was 68% (51% among Caucasians and 94% among blacks). Hypovitaminosis D in these children was associated with greater fat mass and higher body mass index z-scores, measurement during winter, older subjects with darker skin colour and households with lower caregiver education levels. Another contributing factor to low vitamin D status was lower dietary intake of vitamin D (Weng et al., 2007) due to a preference for foods not fortified with vitamin D and also not consuming vitamin D supplements.

In another larger study in the US-NHANES, the majority of children were found to have suboptimal levels (50 to < 75 nmol/L) of 25(OH)D, especially non-Hispanic black and Hispanic children (Mansbach et al., 2009). Some researchers have suggested that maintaining 25(OH)D concentrations above the "sufficiency or optimal" level (>50 nmol/L) throughout the year may be a cost-effective measure of improving bone health especially in adolescent children (Cashman et al., 2008). In general, the prevalence of vitamin D deficiency/hypovitaminosis D in the US is high in African American children, and is accompanied by secondary hyperparathyroidism, which may potentially lead to bone resorption (Gordon et al., 2004). Lack of diets sufficient in vitamin D and seasonal variations in UV exposure may explain the prevalence of this nutritional

deficiency among otherwise healthy children. Hence, Moore et al., 2005 has recommended that "An alternative to greater sun exposure is to increase the availability of fortified foods, supporting the greater use of dietary supplements and encouraging changes in dietary patterns to consume more food fortified with vitamin D should be considered to address this important health issue". However, this may be a problem in families with low socioeconomic status (SES) and with no information on the importance of vitamin D in bone health and disease.

2.9.2.3 Latin America

Few studies have been reported on vitamin D status among different populations in Latin America. Brazil is a Latin American country that falls under a list of countries that have sunshine in abundance (El-Hajj et al., 2001, Thacher et al., 2012, Al-Ghamdi et al., 2012). But unfortunately, it does not escape the effects of hypovitaminosis D. The median population of 25(OH)D in a study of adults (18 – 90 years) conducted in Sao Paulo was reported to be 54 nmol/L and 77% had hypovitaminosis D (< 75 nmol/L). As a result, living in a sunny country does not always guarantee sufficient 25(OH)D in an otherwise healthy population. Another possible cause for hypovitaminosis D suggested by authors was the avoidance of sunlight exposure by using UV protection cream, which is strongly recommended for the prevention of skin cancer by dermatologists (Unger et al., 2010).

In Chile, vitamin D deficiency (< 50 nmol/L) was reported in children living in austral latitudes but surprisingly 25(OH)D had no association with PTH, calcium, and phosphate (Le et al., 2013b) as reported by other researchers (Oliveri et al., 1993, Weng et al., 2007). This vitamin D deficiency picture is similar to what has been reported in Argentina in both the urban and rural communities (Gobbi et al., 2009, Brito et al., 2013).

2.9.2.4 Europe

Europe, as a continent is also reported to have a problem of hypovitaminosis D in varying degrees across its countries (McKenna, 1992). Furthermore, reports in European countries have found that countries located at higher latitudes (46^0N, 56^0N, 64^0N) and without health programs or policies in place for food fortification and supplementation are often at the receiving end of hypovitaminosis D (Kristinsson and Valdimarsson, 1998, Marrone et al., 2012, Thuesen et al., 2012). A study in northern Italy on children (2 – 220 months) has shown vitamin D deficiency (< 37.5 nmol/L) to be common (> 50% of the study population) especially in the winter months.

However, the deficiency was higher in migrant subjects with darker skin colour from India and Africa as compared to their white Italian peers. There is a handful of foods that naturally contain vitamin D and in Italy, there are only a few foods that are fortified. Hence it was emphasised that darker-skinned subjects should be targeted for screening and the caregivers must be educated on the advantages (prevention of bone-related diseases and non-bone related diseases) of exposing children to sunlight (Rovner and O'Brien, 2008, Marrone et al., 2012). Another European country undergoing challenges of vitamin D deficiency due to low vitamin D intake is Austria (48^0N). Vitamin D intakes (2.1 ± 1.4 µg/dl) were reported to be lower in 68% of the total population, and did not meet the dietary vitamin D recommendations. Adolescent children aged 15 -19 years had also shown a high incidence of low vitamin D intake (1.6 ± 1.0 µg/dl, with a vitamin D status of 11.2 ± 8.1 nmol/L) (Koenig and Elmadfa, 2000). A recent study has also found dietary vitamin D to be low across all age ranges and that it has failed to meet dietary reference ranges as recommended by other nutrition societies. (Elmadfa, 2017). Children often had low intake levels (1.39 µg/dl (0.93) in boys and 1.26 µg/dl (1.00) in girls). This was evidenced by 13 - 14 year-old girls who had the lowest median 25(OH)D level (40.2 nmol/L, IQ range: 25.0) while the boys of this age group had a higher median level though not significant (51.6 nmol/L, IQ range: 36.8) (Elmadfa, 2017). However, other European countries have programs of vitamin D fortification and supplementation in place. In a study of Finnish children (62^0N) by Piirainen et al., (2007), results have shown that national fortification of fluid milks and margarines with vitamin D safely improved vitamin D in children during wintertime. Hence, 25(OH)D was higher after fortification (65 (95% CI 59.7 – 70.1) nmol/L) as compared to before fortification (55 (95% CI 51.0 – 58.4) nmol/L; p = 0.002). A similar study in Swedish women (60^0N) has shown vitamin D intake (through fatty fish, vitamin D-fortified reduced-fat dairy products, regular supplement use and taking a sunny vacation) to be directly proportional to 25(OH)D in the winter months (Burgaz et al., 2007). However, errors of vitamin D supplementation were reported by Chirita-Emandi et al., 2015, in Romania where higher mean vitamin D levels were found in the 0 to 2 year old age group (0 - 1 year olds, mean 25(OH)D = 171 nmol/L; 1 - 2 year olds, mean 25(OH)D = 162 nmol/L) compared to older age groups. Based on supplement errors found, it was an eye-opener for the need to increase awareness on the importance of preventing vitamin D supplementation administration errors in young children.

Multicentre studies have also assessed vitamin D in adolescent children. One of the studies is the *OPTImal FORtification* of vitamin D study in adolescent girls (mean age 12.5 years) from 4 northern European countries (Denmark, Finland, Poland and Ireland (51^0N – 60^0N). The prevalence of vitamin D deficiency (25(OH)D < 25 nmol/L), ranged from 26 to 51% while over 90% of the adolescents had suboptimal 25(OH)D levels of < 50 nmol/L (Andersen et al., 2005, Tylavsky et al., 2006). In the *Healthy Lifestyle in Europe by Nutrition in Adolescence* (HELENA) study in nine European countries, almost 80% of 1006 adolescents aged 12.5 – 17.5 years had suboptimal levels; sufficiency was classified as > 75 nmol/L. It was found that 39% of the study population had levels of 50 – 75 nmol/L, 27% were classified as deficient (27.5- 49.99 nmol/L) and 15% as severely deficient (< 27.5 nmol/L). Adolescent males were found to have higher 25(OH)D and higher vitamin D intakes than their female peers (Valtuena et al., 2013). Evidence from literature review in central European populations has concluded that 25(OH)D levels had a mean below 75 nmol/L (Pludowski et al., 2014). Hence proper administration of vitamin D_3 and calcium supplementation may decrease the incidence of bone-related diseases in the young and elderly subjects (Lips, 2001).

2.9.2.5 Asia

Asia is another continent which has not been spared from the harmful effects of hypovitaminosis D even though much of the continent is blessed with an abundance of sunlight. In India, the majority of data published on infants, toddlers and the elderly has reported a high prevalence of vitamin D deficiency and insufficiency irrespective of the cut-off points used by the authors (Agarwal et al., 2002, Harinarayan, 2005, Goswami et al., 2008, Sahu et al., 2009). In a study conducted in rural India, there was significant seasonal 25(OH)D variation and a high prevalence of vitamin D deficiency in pregnant women and adolescent girls. However, boys were found to have significantly higher 25(OH)D levels as compared to their female counterparts [67.5 ± 29.0 (nmol/L) versus 31.3 ± 13.5 (nmol/L), ($p < 0.001$)]. This finding is in line with the Indian tradition which allows boys "greater freedom to be outdoors and have preference in terms of their diet". This tradition impacts negatively on the vitamin D status and bone mass of girls (Sahu et al., 2009). In a similar study in Delhi, India (28^0N) which is regarded as one of the most polluted cities in the world, children were found to be at risk of developing vitamin D deficiency rickets. Vitamin D supplementation or fortification of foodstuffs was suggested as the "magic pill" that would reverse the clinical condition (rickets) (Marwaha et al., 2005, Puri et al., 2008,

Marwaha et al., 2011). Both the urban and rural Indian data show that children, adults and the elderly may suffer from metabolic bone diseases such as rickets, osteomalacia, and osteoporosis as a result of vitamin D deficiency. Other similar studies on hypovitaminosis D were reported in Pakistan (Rashid et al., 1983, Dar et al., 2012, Shah et al., 2014). This high prevalence of low 25(OH)D levels in South Asian countries may be explained in terms of skin pigmentation, traditional clothing, air pollution, and limited outdoor lifestyle.

In general, hypovitaminosis D in the Middle East has been explained by insufficient sun exposure to the skin due to cultural practices and prolonged breastfeeding with no vitamin D supplementation or fortification. The Middle East ($15^0 – 36^0$ N), as part of the Asian continent, receives enough sunlight throughout the year to ensure vitamin D sufficiency in most persons who spend time outdoors. However, sunlight exposure is limited by cultural practices which make its ability to reach the human skin a challenge. A cultural practice (covering of the whole body with clothes) in the Middle East that require immediate attention is about mothers being vitamin D deficient. These mothers are putting their infants at risk of vitamin D deficiency due to prolonged breastfeeding without vitamin D supplementation (Taha et al., 1984).

2.9.2.6 Australasia

New Zealand has also encountered the challenges of hypovitaminosis D, despite the hours of sunshine being enough to produce sufficient vitamin D on the skin (Rockell et al., 2008). A study in New Zealand has found on average, 19% of newborns had 25(OH)D < 25 nmol/L and 57% had levels of less than 50 nmol/L, with only 27% having levels of 75 nmol/L or higher (Camargo et al., 2010), which are levels associated with optimal health in older children and adults (Hill et al., 2006, Holick, 2007a). However, the strongest determinants of 25(OH)D in newborns' cord blood were winter month of birth and non-European ancestry. Another study in children (New Zealand European and other Europeans) aged 12 – 22 months has found approximately 94% of children to have 25(OH)D of > 50 nmol/L in the summer season and 80% of participants sampled in the winter season to have 25(OH)D of < 50 nmol/L. This showed that there is seasonal variation in 25(OH)D levels and it further implies that post-summer vitamin D stores were not enough to maintain 25(OH)D levels of greater than 50 nmol/L (Houghton et al., 2010). The mean concentration of 25(OH)D (52 nmol/L) in children aged 12 – 22 months was similar to that observed in the 2002 National Survey of New Zealand children aged 5 – 14 years (50

nmol/L) (Houghton et al., 2010). The study on 5 - 14 year-old children found that vitamin D deficiency was < 17.5 nmol/L, in 5% (Maori), 8% (Pacific) and 3% (New Zealand Europeans)] and insufficiency (< 37.5 nmol/L, New Zealand, 41% (Maori), 59% (Pacific) and 25% (New Zealand Europeans) in almost all age groups (Rockell et al., 2005, Daly et al., 2012). The prevalence of hypovitaminosis D was reported to increase with age and the disturbing issue was the high risk of compromised growth and development as 25(OH)D concentrations decreased from replete to deficient. At present, there is no universal food fortification with vitamin D in New Zealand and Australia. As a result, the main source of vitamin D is through skin exposure to sunlight for the endogenous synthesis of vitamin D in the body (Vanlint, 2005, Rockell et al., 2008).

Australia is a developed country which has good nutritional and health standards, and has sufficient sunshine (an annual average of approximately 7 hours) for the cutaneous synthesis of vitamin D. The issue is, 95% of skin cancers and 99% of melanomas in Australia are due to sun exposure, hence avoiding skin cancer by applying sunscreen or having limited outdoor activity may lead to vitamin D deficiency and vice versa (Moan et al., 2008).

There have been confirmed case series of rickets (median age 15.1 months) due to vitamin D deficiency of < 20 nmol/L (25(OH)D and confirmed rickets by long bone x-ray changes such as cupping, slaying, and fraying of the metaphysis), which mirrors the immigration trends (Robinson et al., 2006). A study by McGillivray et al., 2007 has found immigrant children (mean and sd 8.9 ± 4.4 years) from East Africa living in Melbourne Australia ($37^0$49 South) to have asymptomatic vitamin D deficiency. Low 25(OH)D levels of < 50 nmol/L occurred in 87% of the studied population and vitamin D deficiency (< 25 nmol/L) was 44%. The risk factor was increased skin pigmentation and the tendency to spend time indoors (McGillivray et al., 2007). There was also a problem in newly arrived refugee children (age range from 0 to 17 years) in Sydney Australia, wherein vitamin D deficiency was the most common diagnosis: 61% had serum 25(OH)D_3 of < 50 nmol/L (Sheikh et al., 2009). Another study has reported vitamin D deficiency (< 25 nmol/L) of 25(OH)D in at-risk African populations in Sydney Australia to be 87%. It was recommended that there must be a screening of vitamin D deficiency and routine vitamin D supplementation in at-risk populations such as dark-skinned or veiled groups (Benitez-Aguirre et al., 2009). Immigrants from other countries were also found to have vitamin D

deficiency in Australia which was related to darker skin colour, culture and indoor lifestyle (Jang et al., 2013).

A position statement by New Zealand and Australian researchers has recommended that the level for serum (25(OH)D) in infants, children, adolescents and during pregnancy and lactation should be 50 nmol/L. This level may need to be 10–20 nmol/L higher at the end of summer to maintain levels of 50 nmol/L over winter and spring. Children over 12 months are requested to consume 600 IU of vitamin D daily in their diet, and during pregnancy and lactation assuming minimal sun exposure (Paxton et al., 2013).

2.9.3 Prevalence of vitamin D deficiency

Vitamin D deficiency (Table 2.5, page 42) is shown to be a worldwide phenomenon and has been described as a pandemic. Despite the difference in cut-offs reported by researchers, vitamin D deficiency has been found in every continent, across all ages, genders, ethnicities and also in countries with an abundance of sunlight. Recently, a systematic review and meta-analysis of studies that measured 25(OH)D levels in healthy participants residing in Africa has showed that the prevalence of vitamin D deficiency is high in the Africa population (Migore et al., 2020).

Table 2.4: Prevalence of vitamin D deficiency across the continents based on various cut-offs of deficiency

Author	Place	Latitude	Vitamin D cut-off	Prevalence	Subjects
		Africa			
Pettifor et al., 1978a	South Africa (Cape Town)	26.2056° S	<25 nmol/L	0%	Children
Meddeb et al., 2005	Tunisia, Tunis (Ariana)	36°4656 N	<37.5 nmol/L	48%	Women
Wejse et al., 2007	West Africa, (Guinea Bissau)	11.8037° N	≤ 50 nmol/L	9% TB patients, 13.2% controls	Adults
Martineau et al., 2011a	South Africa (Cape Town)	33.9249° S	< 50 nmol/L	62.7%	Adults
Sudfeld et al., 2012	Tanzania (Dar es Salaam)	6.3690° S	< 50 nmol/L	9%	Adults on ART
Djennane et al., 2014a	Northern Algeria (Tizi-Ouzou)	36°0.45′ N	< 30 nmol/L	8%	School children
George, 2014	South Africa (Johannesburg)	26.2056° S	< 30 nmol/L	28.6% 5.1%	Indian Adults Black African Adults
Lategan et al., 2016	South Africa (Free State)	29.0852° S	30 – 50 nmol/L	4%	Adults
Velaphi, 2017	South Africa (Johannesburg)	26.2056° S	< 30 nmol/L	18.8% 39.8	Women Newborns
		Asia			

Harinarayan, 2005	South India (Tirupati)	12.2602° N	< 25 nmol/L	30%	Postmenopausal
Hashemipour et al., 2006	Iran (Tehran)	35.6892° N	< 35 nmol/L	81%	Healthy adult women
Harinarayan et al., 2007	South India (Tirupati)	12.2602° N	<50 nmol/L	44% Men 70% Women	Healthy subjects
Puri et al., 2008	India (New Delhi)	20.5937° N	< 50 nmol/L	69%	Children
Goswami et al., 2008	India (New Delhi)	20.5937° N	< 50 nmol/L	94%	Children
Sahu et al., 2009	North India (Barabanki)	8°4' to 37°6' N	< 50 nmol/L	89% adolescents 74% pregnant women	Adolescents and pregnant women
Harinarayan et al., 2011	South India (Tirupati)	12.2602° N	< 50 nmol/L	76%	Reproductive women and postmenopausal women
Neyestani et al., 2012	Iran (Tehran)	35.6892° N	<12.5 nmol/L < 25 nmol/L	38.3% Severe vitamin D deficiency 86% Vitamin D deficiency	School age children
Al-Ghamdi et al., 2012	Saudi Arabia (Jeddah)	23.8859° N	< 25 nmol/L	2.5% (6-9 years) 31% (13-14years) 64% (15-18years)	Children
Khan et al., 2012a	Pakistan (Karachi)	30.3753° N	< 50 nmol/L	92%	Pakistani females

Wang et al., 2017	China (Huzhou Maternal and Child Care Hospital)	49.7448" N	< 50 nmol/L	6%	Children
		Australasia			
Brock et al., 2004	Australian Immigrants (Chinese Middle East Vietnamese)	25.2744° S	<25 nmol/L	28% Chinese 58% Middle East 18% Vietnamese	Elderly subjects
Rockell et al., 2005	New Zealanders (Dunedin) Maori Pacific NZEO	40.9006° S	< 17.5 nmol/L	 5% Maori 8% Pacific 3% NZEO	Children
		Europe			
Lapatsanis et al., 2005	Greece (Northwest of Greece)	37° 59' 1.7160" N	< 25 nmol/L	47%	Children
Harel et al., 2011	Rhode Island (Hasbro's children hospital)	41.5801° N	< 50 nmol/L	53% Female, 47% Male	Adolescents
Andıran et al., 2012	Turkey (Ankara)	38.9637° N	≤ 37 nmol/L	25%	Children and adolescents
Eggemoen et al., 2013	Norway (Immigrants from Africa and Asia)	60.4720° N	< 50 nmol/L	80%	Children, adolescents and adults

Voortman et al., 2015	The Netherlands (Rotterdam)	52.1326° N	< 25 nmol/L	23.6%	School children
		North America			
Mansbach et al., 2009	USA (NHANES)	37.0902° N	< 25 nmol/L	1%	Children
Harkness and Cromer, 2005b	USA (Greater Cleveland Ohio)	41° 29 58 N	< 27.5 nmol/L	17%	Adolescent females
Saintonge et al., 2009	USA (NHANES)	37.0902° N	27.5 to < 50 nmol/L	2% to 14%	Children
Rajakumar et al., 2011	USA (Boston)	42.3601° N	< 50 nmol/L	73% Black 40% Whites	Children
Coney et al., 2012	USA (Nashville Tennessee)	36.1627° N	< 50 nmol/L	98% Black 45% Women	Women
Au et al., 2013	USA (NHANES)	37.0902° N	< 50 nmol/L	36%	Racially diverse children
		South America			
Tau et al., 2007	Argentina (Ushuaia, Tierra Del Fuego)	38.4161° S	< 25 nmol/L	0%	Children
Gobbi et al., 2009	Argentina(Cordoba province)	38.4161° S	< 50 nmol/L	33%	Women
Villamor et al., 2011	Colombia (Bogota)	4.5709° N	< 50 nmol/L	11.6%	Children

Le et al., 2013a	Chile (Coyhaique)	45.3551° S	< 50 nmol/L	64%	Pre-School children
Brito et al., 2013	Argentina (Buenos Aires City)	38.4161° S	< 50 nmol/L	88%	Women

2.9.4 Vitamin D fortification and supplementation

Vitamin D fortification of foods and supplementation have been recommended as methods to reduce the prevalence of vitamin D deficiency (Agarwal et al., 2002, Moore et al., 2005, Allali et al., 2009, Al-Ghamdi et al., 2012, Le et al., 2013a). Based on the hypovitaminosis D prevalence in the US (Buffington et al., 1993, Harris, 2006, Wortsman et al., 2000, Arunabh et al., 2003), the US Department of Agriculture has recommended a list of foodstuffs to be consumed that naturally contain vitamin D and foodstuffs that are fortified with vitamin D (Table 2.5) in order to eradicate the vitamin D deficiency epidemic.

Table 2.5: Examples of foods containing vitamin D, either naturally or through fortification.

Food	**Amount of vitamin D**
Fatty fish (e.g. Canned pink Salmon herring, Canned Mackerel, Canned Sardines)	2.2 – 13.7 μg per 100g (86 – 547 IU)
Portobello and Shiitake mushrooms	0.2 - 0.4 μg per 100g (10 – 18 IU)
Liver (Beef)	1.2 μg per 100g (49 IU)
Eggs (Large, 50g)	1.1 μg per egg (44IU)
Fortified foods (e.g. Margarine, milk and yoghurts)	5 – 10 μg per litre (200 – 400IU)

Footnote: 1 μg = 40 IU

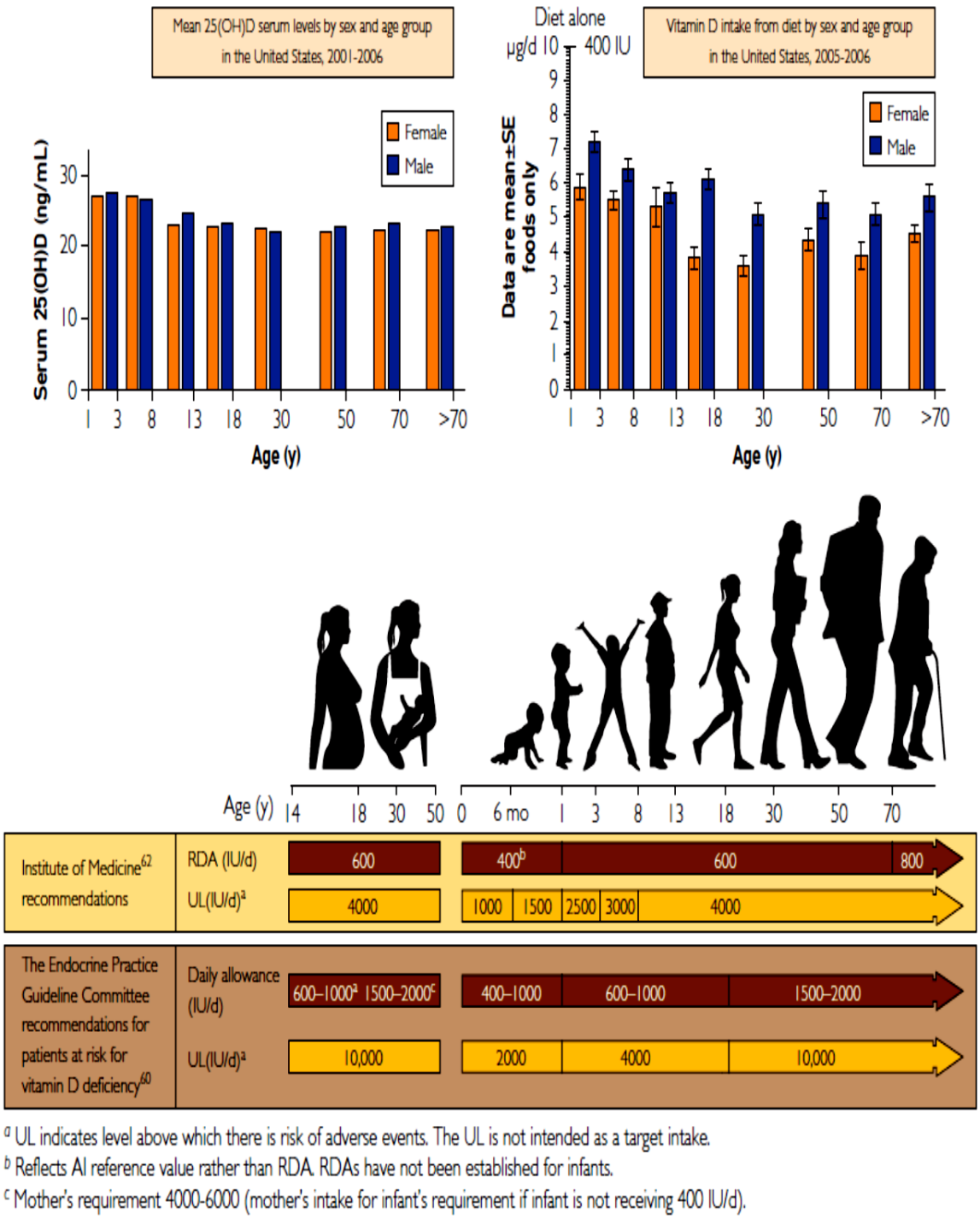
Source: US Department of Agriculture, Agricultural Research Service, USDA, 2012.

USDA National Nutrient Database for standard reference. Release 25 Nutrient data laboratory Home page. URL: www.ars.usda.gov/ba/bhnrc/nd/(accessed 15 Feb 2013. Note that the recommendation pertains to US foods, and may not be appropriate in other countries.

In making health practitioners aware of the consequences of hypovitaminosis D, the Institute of Medicine (US) and the Endocrine Practice Guidelines Committee have established recommended vitamin D adequate intakes for all age ranges in order to prevent hypovitaminosis D (Figure 2.4, page 49).

The report estimated that children over 1-year-old need at least 600 IU of vitamin D a day, with a maximum upper limit of 2500 IU for children aged 1 to 3 years, 3000 IU for children from 4 to 8

years old, and 4000 IU/day for children aged 9 or more years old (Ross et al., 2011a). The current Guidelines from the Section on Breastfeeding and the Committee on Nutrition of the American Academy of Pediatrics recommended a minimum daily intake of 400 IU of vitamin D for all infants, children, and adolescents, starting soon after birth (Wagner and Greer, 2008, Pediatrics, 2012). On the other hand, some researchers (Holick, 2010, Holick, 2011) estimated that teenagers and adults need at least 2000 IU of vitamin D a day to meet their body's requirements. Concerning serum levels, the 2011 IOM Committee targeted a serum level of at least 50 nmol/L of 25(OH)D as meeting the needs of nearly all children, in agreement with the Pediatric Endocrine Society (Misra et al., 2008).



[a] UL indicates level above which there is risk of adverse events. The UL is not intended as a target intake.
[b] Reflects AI reference value rather than RDA. RDAs have not been established for infants.
[c] Mother's requirement 4000-6000 (mother's intake for infant's requirement if infant is not receiving 400 IU/d).

Figure 2.4: Vitamin D intakes as recommended by Institute of Medicine (US) and the Endocrine Practice Guidelines Committee. Source: Hossein-nezhad and Holick, 2013.

Figure 2.4 depicts the recommendations of the Institute of Medicine and the Endocrine Society for vitamin D requirements in order for the public to attain "sufficient" or "optimal" 25(OH)D level (Holick et al., 2011, Ross et al., 2011a). The IOM report focuses on the bone health level as

it found no evidence that a serum 25(OH)D of greater than 50 nmol/L had beneficial effects at a population level. However, the Endocrine Society has considered that vitamin D has functional activity beyond skeletal function. Hence, it has recommended that serum 25(OH)D levels of 75 nmol/L should be attained in order to avoid non-skeletal diseases that may be associated with low vitamin D status.

2.9.5 Consequences of vitamin D deficiency

Until recently, the only known consequence of vitamin D deficiency was related to bone and mineral metabolism, but there has been an emergence of evidence which suggests that vitamin D deficiency may also be associated with non-bone related diseases (Farrag et al., 2017, Gong et al., 2017), as illustrated in figure 2.5 (page 51).

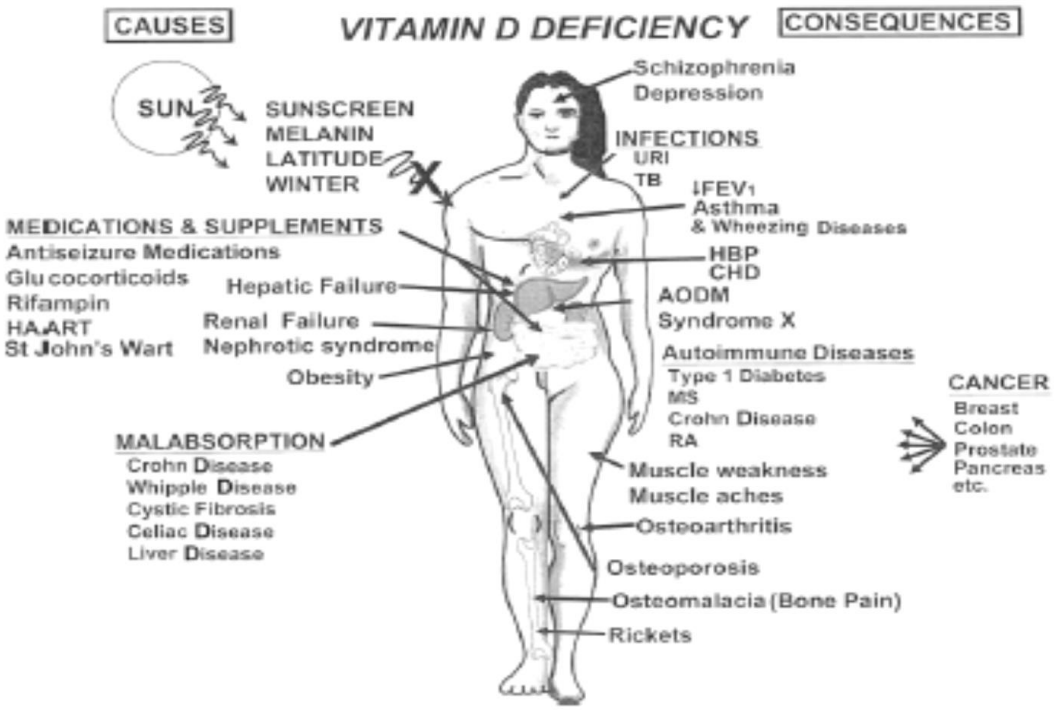


Figure 2.5: Major causes of vitamin D deficiency and potential health consequences. HBP - High blood pressure, AODM – adult onset diabetes mellitus, CHD - coronary heart disease, FEV - forced respiratory volume, HAART - highly active antiretroviral therapy, MS - multiple sclerosis, RA - Rheumatoid Arthritis, Tb - tuberculosis, UTI - urinary tract infection.

Source: Wacker and Holick, 2013.

2.9.5.1 Rickets and Osteomalacia

Vitamin D deficiency results in a disease condition called rickets in children. This is a disease of bone mineralization in childhood which results in soft bones that bow under the weight of the body (Wharton and Bishop, 2003), due to the mineralization defect at the epiphyseal growth plates and bone tissue. Vitamin D deficiency in adults leads to osteomalacia which is a mineralization defect of the bone tissue after the closure of the growth plate (Soliman and Kalra, 2013).

2.9.5.2 Hypertension

Hypertension is a worldwide phenomenon, with higher prevalence in blacks compared to their Caucasian counterparts and other ethnic groups (Van Rooyen et al., 2016, Mokwatsi et al., 2017, Swart et al., 2017). Its prevalence is inversely associated with vitamin D status (Forman et al., 2007, Rostand, 2014). This was also reported 30 years ago when an inverse association between

25(OH)D, 1,25(OH)2D and plasma renin activity in hypertensive patients was described (Resnick et al., 1986). Evidence shows that in humans, skin exposure to UVB has been linked to a decrease in blood pressure (Kuneš et al., 1991). However, this association was not found in other studies (Lind et al., 1995, Jorde et al., 2010a). An experimental study by Weng et al., 2013 has emphasized the relationship by reporting that apolipoprotein E-null mice, when given a diet lacking vitamin D, develop increased systolic and diastolic blood pressure as well as increased plasma renin activity that is reversed by a vitamin D sufficient diet. Furthermore, laboratory animal experiments have implicated 1,25-dihydroxyvitamin D in inhibiting renin expression in the juxtaglomerular apparatus and blocking the proliferation of vascular smooth muscle cells (VSMC), influencing systemic blood pressure (Li et al., 2002, Abolfotouh et al., 2012).

2.9.5.3 Dyslipidaemia

Studies have associated abnormal lipid profiles with atheromatosis, a condition in which the arterial wall becomes thick and hard as a result of the accumulation of plaque leading to heart attack (Duff and McMillan, 1951, Chambless et al., 1997). Another known predictor of cardiovascular diseases (CVD) is obesity. Furthermore, overweight (BMI 25–29.9 kg/m^2) and obesity (BMI ≥30 kg/m^2) are often related to unfavourable lipid profile (Szczygielska et al., 2003). 25(OH)D levels have been associated with dyslipidaemia in children (Kumar et al., 2009, O'Donnell, 2015) and adults (Wang et al., 2016). In US children and adolescents aged 1 – 21 years (adjusted for age, sex, and ethnicity), 25(OH)D deficiency was associated with cardiovascular risk factors which included high blood pressure, low HDL cholesterol and high PTH (Kumar et al., 2009). But, in another US study involving adolescents (adjusted for age, ethnicity, socioeconomic status and waist circumference), higher calcium levels predicted increased cardiovascular risk rather than low 25(OH)D levels or high PTH levels (Williams et al., 2011). The authors, however, cautioned that their findings required replication in prospective studies. In Finnish prepubertal children (6 to 8 years) higher serum 25(OH)D was associated with lower plasma total cholesterol (TC), LDL cholesterol, HDL cholesterol, and triglycerides. However, after adjusting for confounders, the associations with triglycerides disappeared (Soininen et al., 2018). Low serum 25(OH)D in obese children has been associated with abnormalities in the lipid profile with hypertriglyceridaemia (Rodríguez-Rodríguez et al., 2011) and low HDL-C (Johnson et al., 2010, Rajakumar et al., 2011). The problem in the majority of studies is that they are cross-sectional in design, with only a handful of longitudinal data reported (Jorde et al., 2010b, Williams et al., 2014, Faridi et al., 2017). In a study by Jorde

et al. (2010b), a longitudinal association of serum 25(OH)D with triglycerides in adults over a 14 year period (after adjusting for age, sex, and BMI) explained the relation between lower serum 25 (OH)D levels and mortality. In another adult study, vitamin D deficiency was associated with lower HDL cholesterol and higher TC to HDL cholesterol ratio over a 5 year period after adjusting for demographic and lifestyle factors (Faridi et al., 2017). More evidence from clinical trials is warranted to better assess how 25(OH)D may affect lipids and thereby possibly influence cardiovascular risk. In Avon longitudinal study in children, higher $25(OH)D_3$ levels were associated with cardioprotective levels of HDL-C, Apo-A1 and adiponectin. But association of low $25(OH)D_3$ with cardiovascular risk factors was a mixed bag (low levels of inflammatory markers, higher Apo-A1 and higher triglycerides) (Williams et al., 2014). It was further suggested that associations in larger prospective studies is warranted. However, a systematic review and meta-analysis of prospective studies have suggested that vitamin D supplementation may act to protect against CVD through improving risk factors that include hypertension, elevated PTH, dyslipidaemia and inflammation (Grandi et al., 2010, Wang et al., 2012).

2.9.5.4 Insulin resistance, and diabetes mellitus

Although diabetes mellitus (type I and II) has been recognized for quite some time (O'Donnell, 2015, Zaccardi et al., 2015), its prevalence is increasing due in large part to nutritional transition and populations being exposed to high fat and sugar diets with less physical activity. The prevalence of diabetes mellitus for all ages was estimated to be 2.8% in 2000 and projected to be 4.4% by 2030 (Wild et al., 2004).

The relation between low vitamin D status and insulin resistance has been reported (Norman et al., 1980, Cade and Norman, 1986), and is now receiving increased attention (Scragg et al., 2004, Chiu et al., 2004). Hypovitaminosis D has been suggested to be a risk factor for reduced insulin secretion, impaired glucose tolerance, and non-insulin dependent diabetes mellitus (NIDDM) in adults (Norman et al., 1980, Cade and Norman, 1986, Boucher et al., 1995, Baynes et al., 1997a, Baynes et al., 1997b, Chiu et al., 2004, Scragg et al., 2004, Talaei et al., 2013, Grammatiki et al., 2018) and type I diabetes in children and adolescents (Janner et al., 2010, Ziaei-Kajbaf et al., 2018, Akdere et al., 2018). Studies have also shown vitamin D deficiency to be associated with type I diabetes (Greer et al., 2007, Svoren et al., 2009, Janner et al., 2010), which is regarded as an autoimmune disease (Bach, 1994). Vitamin D is believed to inhibit the autoimmune response targeted towards the β cells of the pancreas. There is evidence that vitamin D might influence

glucose concentrations through its action on vitamin D receptors found in the pancreas (Takiishi et al., 2010). It has been shown that in both human and animal studies, vitamin D plays an important role in normalizing insulin release in response to glucose and for maintenance of glucose tolerance (Orwoll et al., 1994, Palomer et al., 2008).

Controversies still exist concerning the relationship between vitamin D and insulin as some studies have failed to show any relationship between 25(OH)D and insulin resistance in obese children (Reinehr et al., 2007, Smotkin-Tangorra et al., 2007) and adults (Del Gobbo et al., 2010). Some reports have found that the relationship between 25(OH)D concentration and estimates of insulin secretion disappears when adjusted for the differences in the degree of adiposity (Del Gobbo et al., 2010, Muscogiuri et al., 2010, Chacko et al., 2011), hence these findings may suggest that adiposity has a greater influence than vitamin D on insulin status.

In supplementation studies with vitamin D, healthy subjects with initially insufficient serum 25(OH)D levels had no improvement in insulin sensitivity or secretion or in serum lipid profiles (George et al., 2012). Also in a study on diabetic subjects, Orwoll et al., 1994 reported no benefit in giving vitamin D supplements. Researchers have suggested that prevention of hypovitaminosis D in groups at high-risk of NIDDM warrants further study as it may contribute to maintaining glucose tolerance and avoidance of the decline into NIDDM (Baynes et al., 1997a). In a larger study, the possible role of vitamin D in diabetes was shown in healthy women (N= 83779), with no history of diabetes over 2 - 4 years. In this study, a combined daily intake of greater than 1200 mg calcium and greater than 800 IU vitamin D was associated with a 33% lower risk of type 2 diabetes as compared to a daily intake of less than 600 mg of calcium and less than 400 IU of vitamin D (Pittas et al., 2006).

In further assessing the association between vitamin D status and diabetes, He et al., 2018 conducted a systematic review and meta-analysis on vitamin D supplementation in non-diabetic subjects. The authors found no significant effect of vitamin D on controlling fasting plasma glucose, improving insulin resistance or prevention of diabetes mellitus type 2 in non-diabetic subjects. However in a stratified analysis, vitamin D supplementation had differential effects on fasting plasma glucose (FPG) control, insulin sensitivity improvement and type 2 diabetes mellitus prevention in individuals with different baseline states: fasting plasma glucose decreased in individuals with a BMI of < 25 kg/m^2 ($p = 0.048$), insulin resistance was improved for individuals with 25(OH)D of ≥ 75 nmol/L ($p = 0.021$) and risk of type 2 diabetes diabetes

mellitus was lowered for pre-diabetic individuals ($p = 0.047$) of individuals with BMI of ≤ 25 to < 30 kg/m^2 ($p = 0.047$). A similar investigation in a systematic review and meta-analysis has reported that there is currently insufficient evidence of the beneficial effects on vitamin D supplementation as a way of improving glycaemia or insulin resistance in individuals with diabetes mellitus, normal fasting insulin or improved glucose tolerance (George et al., 2012). Furthermore, clinical trials should focus on individual baselines and supplementation strategy of vitamin D in the prevention of type 2 diabetes.

In conclusion, the relationship of vitamin D with metabolic variables is still subject to much research. Some studies did not show any relationship between insulin in children and adults, while others have found an association. Supplementation of vitamin D has reported no benefits in subjects with NIDDM and no NIDDM, but other studies have found a reduction in the risk of diabetes mellitus. Hence, consensus on optimum vitamin D status required to prevent the risk of non-bone diseases is still an on-going discussion by the researchers.

2.9.5.5 Autoimmune diseases and cancers

The role of vitamin D deficiency in the development of autoimmune diseases has been reported over the past 25 years (Kröger et al., 1993). Some studies have found an association between vitamin D deficiency and autoimmune diseases such as rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus (Cutolo et al., 2007, Doria et al., 2008). Impairment of immune system functioning by a suboptimal vitamin D status in infancy has been suggested to have long term effects on immune responses later in life (Hyppönen et al., 2007), predisposing subjects to autoimmune diseases and cancers.

Common autoimmune diseases are multiple sclerosis (MS), rheumatoid arthritis (RA), Crohn disease, type I diabetes and systemic lupus erythematosus (SLE). These clinical conditions result from abnormal activation of the immune system, whereby the immune response is directed against harmless self-antigens. The end results are inflammation, tissue damage, and loss of function of the affected organs or joints (Dankers et al., 2017).

2.9.5.6 Immune system and infections

The association between vitamin D deficiency and infectious disorders has been suggested for more than 100 years. Prior to the introduction of anti-tuberculosis drugs, infected persons with TB were treated in sunny mountainous locations in the open, relying on the endogenous production of vitamin D to promote healing of TB (Chesney, 2010, Hart et al., 2011). The major

role of the immune system is in combating foreign organisms by halting their spread in the host and ultimately clearing them from the body. However, pathogen elimination from the body is extremely complex and relies on an elaborate and dynamic communication network using soluble mediators (i.e. cytokines). These assist billions of cells that patrol the body and interact with antigens in order to deal with all pathogens (Dempsey et al., 2003).

The body defence mechanism is comprised of T-lymphocytes, B-lymphocytes, neutrophils and antigen presentation cells (APC) such as monocytes, macrophages and dendritic cells. The discovery of VDR in these cells gave researchers the idea that vitamin D may play a crucial role in the regulation of immune response (Baeke et al., 2010). Furthermore, these immune cells (T helper cells) express the mitochondrial vitamin D-activating enzyme, 1-α-hydroxylase (CYP27B1) and thus play a role in converting 25(OH)D to $1,25(OH)_2D$. In-vitro studies showed that $1,25(OH)_2D$ shifts a cluster of differentiated (CD4) T-cells and major histocompatibility complex (MHC) class II molecules to a more anti-inflammatory profile. $1,25(OH)_2D$ inhibits CD4 T-cells from developing a Th1 cytokine profile tumour necrotic factor-alpha (TNF-α) and interferon-gamma (IFN-γ). Also, it promotes the expression of T-reg cells (secreting transforming growth factor-beta (TGF-β) and interleukin 10 (IL-10) and T-helper cells (Th-2) (producing Interleukin - 4, interleukin - 5, interleukin - 13) (Smolders et al., 2008).

This immunological process is regulated by circulating levels of 25(OH)D which induces the activation of specific toll-like receptors (TLRs) which act as pathogen detectors (Bikle, 2009). TLRs are single, membrane-spanning, non-catalytic receptors that recognize structurally conserved molecules derived from pathogens and activate the innate immunity through cells such as monocytes, macrophages, natural killer cells (NKC) and dendritic cells (DCs) (Lang et al., 2013). Hence, researchers have suggested that $1,25(OH)_2D$ could play important roles in both innate and adaptive immune responses as shown in figure 2.6.

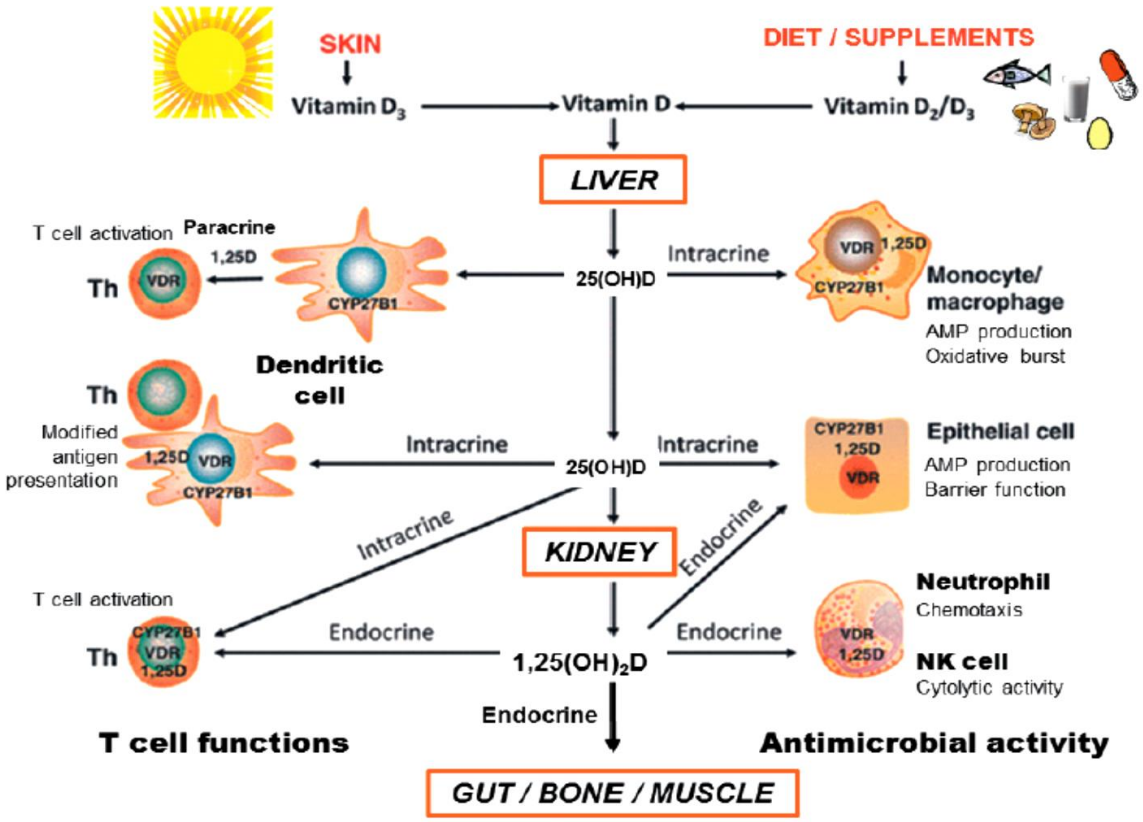


Figure 2.6: Mechanisms for innate and adaptive immune responses to vitamin D.
Source: He et al., 2016.

2.9.5.7 Tuberculosis (TB)

Tuberculosis is a disease associated with a weak immune system and is caused by the bacterium *Mycobacterium tuberculosis*, which may affect different organs, but usually involves the lungs. The vitamin D receptor (VDR) is one of the most frequently studied genes with respect to pulmonary tuberculosis (PTB) susceptibility but the outcome of results has been conflicting based on study sample sizes (Bellamy et al., 1999, Babb et al., 2007, Ates et al., 2011). Studies have reported low 25(OH)D levels to be linked to the presence of TB, even in those countries with plenty of sunlight (Sloan et al., 2015, Smith et al., 2016, Sharma et al., 2017). This has been attributed to the choice of lifestyle such as spending more time indoors and covering the skin with more clothes. On the contrary, a study from The Gambia found higher (mean 62.5 nmol/L) 25(OH)D levels in TB patients as compared to non-TB patients (50.8 nmol/L) (Owolabi et al., 2016). Similar results were obtained from Zimbabwean patients; hence it was suggested that prospectively designed studies with larger sample sizes were warranted to further interrogate the relationship between 25(OH)D levels and TB (Musarurwa et al., 2017).

2.9.5.8 Respiratory infections and Asthma

There is an increasing body of evidence to support the hypothesis that infections carry a greater morbidity in asthmatic subjects than in the healthy population (Jackson and Lemanske, 2010). Studies have also found causality in terms of low 25(OH)D levels and respiratory tract infections especially in children with respiratory diseases such as asthma (Ginde et al., 2009, Jartti et al., 2010).

In a systematic review conducted on 23 observational studies, it was found that vitamin D levels were significantly lower in children with asthma compared to asthma-free children (Jat and Khairwa, 2017). However, the relationship of 25(OH)D levels with incidence or prevalence of asthma, lung function and asthma control gave contradictory results in studies (Gupta et al., 2011, Menon et al., 2012, Krobtrakulchai, 2012). Some researchers are suggesting that vitamin D deficiency and asthma may be linked to reverse causation because vitamin D is synthesized by sun exposure and there is high possibility that children with asthma may spend much of their time indoors due to their asthmatic condition. This is explained by the fact that families want to keep their children at home for fear of an asthma attack if they went outdoors or engaged in physical activities (Uysalol et al., 2013). However after adjusting for factors such as ethnicity and time spent outside the house, there was still a significant association between vitamin D and asthma exacerbations. Hence, reverse causation may be less plausible (Brehm et al., 2012).

On the contrary, there are also some reports suggesting that vitamin D supplementation can instead be a risk factor for asthma and other atopic disorders (Wjst, 2006). In a birth cohort study in Finland, for example, subjects regularly given vitamin D supplementation in the first year of life (about 200 IU/day) had a marginally significantly higher risk of asthma, atopy, and allergic rhinitis at 31 years of age than supplemented controls (Hyppönen et al., 2004).

There is still no consensus among the studies with respect to vitamin D status and incidence or prevalence of asthma. Therefore according to Jat and Khairwa, 2017 “large well-conducted randomized trials of Vitamin D supplementation with different doses and different duration of supplementation to assess the efficacy of Vitamin D on lung function and asthma control in children is warranted”.

2.9.5.9 Neurological diseases

One of the roles of vitamin D that is beyond the bone function is reported to be in the regulation of brain development and its function. This involves inducing expression of synapse structural proteins, neurotrophic factors, and deficient neurotransmitters in neurodegenerative diseases (Mpandzou et al., 2016). In addition, increasing evidence has highlighted the impact of vitamin D deficiency as a favouring factor in various central or peripheral neurological diseases, especially multiple sclerosis and several neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease (Török et al., 2013, Littlejohns et al., 2014, Burton and Costello, 2015). Studies on clinical trials on vitamin D supplementation have stressed the role of vitamin D as a protective and/or prognostic factor in the onset and progress of such neurological conditions (Jorde et al., 2008, Pirotta et al., 2015).

2.9.6 Summary of the introduction and gaps in research

Researchers have considered vitamin D the "sunshine vitamin" because sunshine plays a critical role in maintaining vitamin D sufficiency in the majority of the world's population. Only a small fraction is normally obtained through diet (vitamin D_2 or D_3) from food naturally rich in vitamin D, fortified food or from supplements. However, factors such as latitude, atmospheric pollution, seasons of the year, body fat content, lifestyle factors, dietary intake, and skin colour play a pivotal role in vitamin D status of populations across the globe.

The major function of vitamin D is to facilitate the absorption of calcium and phosphorus in the GIT for the prevention of bone diseases (rickets, osteomalacia and osteoporosis) by enhancing skeletal mineralization. Recent published data have established that vitamin D has functions beyond the bone which involve differentiation, proliferation and cellular function; hence these are referred to as the non-classical functions of vitamin D. Vitamin D receptors are found in many tissues in the body other than the bone and small intestine. As a result, vitamin D deficiency has been linked to diseases that are not bone related such as TB, hypertension, type I and II diabetes mellitus, autoimmune diseases, neurological diseases, cancers, respiratory infections, and asthma.

The challenge in vitamin D research has for decades been around the definition of vitamin D deficiency, insufficiency, and sufficiency (replete). The IOM and Endocrine Society uses different cut-offs for these definitions. The IOM defines vitamin D sufficiency based on maintaining normal musculoskeletal functions and is $\geq$ 50 nmol/L (20ng/ml). Some researchers have suggested that 25(OH)D below 30 nmol/L should be considered to be related to an increased risk of rickets or osteomalacia, and 25(OH)D concentrations between 50 – 125 nmol/L are regarded as safe and sufficient in the general population for skeletal health. In order to bridge knowledge gaps in defining hypovitaminosis D, it will suffice to have an international study on rickets as a multifactorial disease.

The Endocrine Society, on the other hand, defines vitamin D sufficiency as $\geq$ 75 nmol/L (30ng/ml) in an attempt to cover functions that incorporate the prevention of the diseases that are not bone-related. Another thorny issue is the variability in assay methodologies among laboratories measuring vitamin D status ($25(OH)D_2$ and $25(OH)D_3$) because vitamin D is a "difficult" analyte to measure. The inability to standardize or harmonize measurement of vitamin

D metabolites will continue to cause confusion in vitamin D research. Hence, there has been a call for vitamin D standardization.

Irrespective of the assay and definition used to assess vitamin D status, the prevalence of hypovitaminosis D is high across the world. Based on the importance of vitamin D status for the body function, there has been a recommendation for food fortification with vitamin D and for the use of vitamin D supplementation in order to reduce the prevalence of hypovitaminosis D but it is not practiced in every country. Lack of adherence to the recommendations and changes in lifestyle, such as a reduction in outdoor activities and less exposure of the skin to UV radiation has been reported to be associated with vitamin D deficiency in the majority of the world populations. On the public health level, governments across the world are spending billions of US dollars in the treatment of non-communicable diseases such as diabetes mellitus, hypertension, neurological diseases, and more, which are associated with hypovitaminosis D. Studies (systematic review and meta- analysis) on vitamin D supplementation have reported that vitamin D supplementation is important in the prevention or delaying the onset of NIDDM in pre-diabetic subjects, type I diabetes and other diseases. Contrary to these findings, some reports have found vitamin D supplementation to be a risk factor for diseases such as asthma and atopic disorders. However researchers have suggested that more randomised prospective trials using large sample sizes are still warranted.

The majority of studies have reported vitamin D status in various ethnic and gender groups at one point only, with only a few studies conducted longitudinally. There is a lack of data concerning studies involving vitamin D tracking in children, and thus this area warrants further research. There has been an emergence of evidence on the association of vitamin D status with diseases that are not associated with bone health but a shortfall has been that the studies were of a cross-sectional nature. This may pose some doubts because of the factors associated with vitamin D status. Vitamin D supplementation has been reported to be useful in the correction of hypovitaminosis D. The usefulness of vitamin D supplementation has been reported to be in the prevention of noncalcaemic diseases, but not all studies concur. Sufficient vitamin D status has been reported to be associated with low risk of infectious disease but other studies conducted closer to the equator have reported a higher prevalence of vitamin D sufficiency with infectious diseases. The story of vitamin D will continue to unfold even after many decades of research and more revelations will surely follow as further knowledge regarding this intriguing molecule

emerges. More prospective randomised clinical trial studies with larger sample size are warranted to solve this puzzle of vitamin D and non-calcaemic diseases.

Due to unanswered questions in vitamin D research, the present study assessed vitamin D status cross-sectionally in 10-year-old children in an urban setting of Soweto (South Africa) and the leafy suburbs of Johannesburg. Vitamin D has been reported to be influenced by a plethora of factors. Unlike other studies that have assessed vitamin D cross-sectionally, the present study tracked vitamin D status in adolescents of the Bone health cohort of Birth to Twenty children over a period of 10 years. The non-calcaneus association of vitamin D with anthropometric measures and lipid profile was longitudinally assessed in the Bone health cohort of Birth to Twenty children.

CHAPTER 3: METHODOLOGY

3.1 The study cohort background

The group of children used in this study were participants in the Birth to Twenty longitudinal study, which comprised of all childbirths during a 6-week period from 23 April to 8 June 1990. The study subjects are literally called "Mandela's children" because this was the time when the late President Mandela was released from prison. Data collection of children started from birth to when they were ten years old; hence the initial study was called Birth to Ten. The data continued to be collected past the age of ten years until the subjects were twenty years old (Birth to Twenty study) and beyond (Birth to Twenty plus study). This was thanks to the research sponsors such as Wellcome Trust (United Kingdom), Anglo American Chairman's Fund, Medical Research Council of South Africa and Human Science Research Council (HSRC). The Birth to Ten/Birth to Twenty programme is a longitudinal study of child health and development of 3273 children in the Greater Johannesburg Area of South Africa since their birth in 1990. The figure (page 64) shows that the study participants were from Gauteng province in South Africa. Racially, the study subjects are black and white children of the greater Johannesburg-Metropolitan area. In terms of their location of residence, the black children were all from the Soweto townships and white children were from the leafy suburbs in Johannesburg (Richter et al., 2007). This setting was established by the past regime (Apartheid Government), with racial integration taking place since 1990, but the vast majority of the black population still lives in Soweto (Zingoni et al., 2009), under conditions of low socio-economic status.

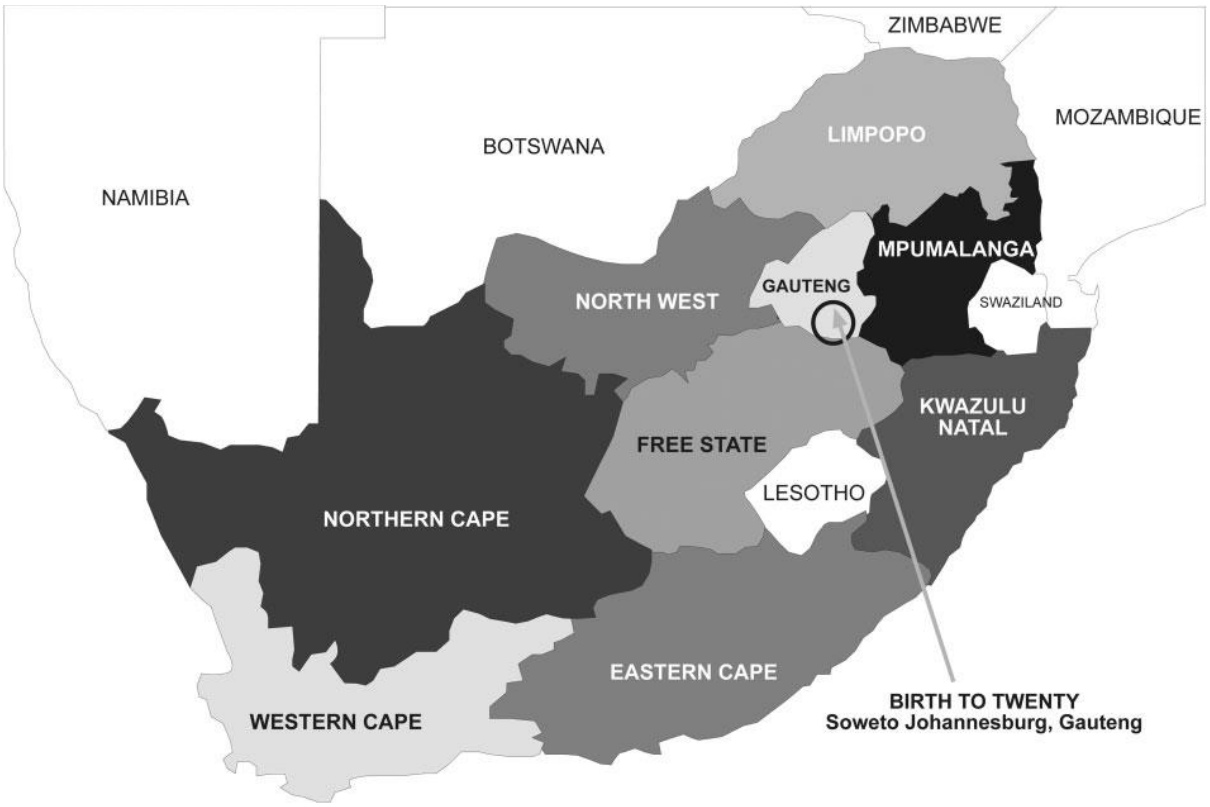


Figure 3.1 Map of South Africa with particular reference to Soweto-Johannesburg, Gauteng

Source: Richter et al., 2007.

Data was collected with the help of Birth to Ten/Twenty staff members (Research assistants, Researchers, Registered Nurses and Laboratory Technicians). The Birth to Ten/Twenty staff members were intensively trained by experts in the field to ensure that they were competent in data collection and storage. The following data was collected for the entire cohort of Birth to Twenty:

1. Anthropometric Data
2. Dietary Data
3. Physical Activity Data
4. Socio-economic Data
5. Biological specimen data (Blood and urine samples)
6. DXA (Bone mass variables)
7. PQCT (Bone mass variables)
8. Growth and psychosocial development

3.2 Data collection

The data collection for the present study subjects was in year 10 for the assessment of vitamin D status. Data for year 11, 13, 15, 17 and 20 was collected to assess vitamin D tracking. The association of vitamin D status with non-communicable disease risk was assessed in year 11, 12, 13, 15 and 18-20. Data collection procedures are written in detail in Chapters 4, 5 and 6.

3.3 Exclusion criteria

Exclusion criteria used in the selection of the study participants was based on anything that is known to affect the bone mass as well as biochemical bone homeostasis such as:

- Chronic illness
- Medication or drugs
- Mineral supplements
- Not assenting to blood taking
- Not willing to give blood
- Difficult veins
- Being a member of church or practicing culture that does not allow blood giving

3.4 Anthropometric measurements and body composition for the entire Birth to Twenty study

The height of the study participants was measured in millimetres using a wall-mounted stadiometer (Holtain, Crymych, UK) and weight in kilograms using a calibrated digital electronic instrument (Dismed, Quebec, Montreal, Canada). In order to control the effects of clothes on weight, the participants were asked to wear minimal clothing provided by the study cohort when being weighed. In assessing children's obesity status and body composition, the BMI was calculated by the formula: body mass (kilograms) divided by height (metres) squared (kg/m^2). Total fat mass and lean mass were measured by dual X-ray absorptiometry, using a Hologic QDR 4500 instrument (Hologic Inc., Bedford, MA, USA).The DXA data were analysed with the software supplied by the manufacturer (Version 11.2).

3.5 Blood specimen collection and laboratory procedures

Fasting blood samples were collected on a yearly basis with the help of registered nurses. In all the biochemical data collected for the Birth to Twenty study, tests for accuracy and precision of various biochemical assays were calculated as the coefficient of variation (CV) using the formula: CV(%) equals to the standard deviation (SD) divided by the mean of the analyte multiplied by a hundred percent [CV(%) = SD/Mean of the Analyte x 100%]. In following the Good Laboratory Clinical Practice (GLCP), our laboratory coefficients of variation were all less than 10 % (**Table 3.1**).

Table 3.1: Inter- and intra-assay coefficients of variations of assay standards

Variable	VitD (low)	VitD (High)	TC (low)	TC (High)	Trig (low)	Trig (High)	HDL-C (Level1)	HDL-C (Level2)	HDL-C (Level3)	LDL-C (Level1)	LDL-C (Level2)	LDL-C (Level3)
Inter (%)	7.98	6.12	3.65	4.47	5.55	5.49	4.46	4.40	3.38	4.70	5.63	4.05
Intra (%)	5.13	2.79	2.95	4.21	4.98	3.95	3.96	3.50	2.5	3.41	4.25	3.76

The table below shows vitamin D definitions used in thesis chapters.

Table 3.2: Vitamin D definitions as per respective papers presented in the thesis

Paper 1 : Chapter 4	Factors influencing the vitamin D status of 10- year-old urban South Africa children.		
Definition	Deficiency	Insufficiency	Sufficiency
Vitamin D categories **Holick, 2007** **Holick 2009**	**< 50 nmol/L**	**50 – 74 nmol/L**	**≥75 nmol/L**
Paper 2 : Chapter 5	Does Vitamin D track through adolescence?		
Definition	Deficiency	Insufficiency	Sufficiency
Vitamin D categories **Dawn-Hughes et al., 2005** **Lee et al., 2007**	< 30 nmol/L	30 – 50 nmol/L	> 50 nmol/L
Paper 2 : Chapter 6	Is vitamin D status associated with non-communicable disease risk in children? A cohort study		
	Deficiency	Insufficiency	Sufficiency
Vitamin D categories **Lips, 2004** **IOM, 2011**	< 50 nmol/L		≥ 50 nmol/L

3.5 Standardization of 25(OH)D assay

Vitamin D status has been defined differently by various researchers due lack of consensus. Hence, researchers in the vitamin D field have suggested vitamin D standardization as way of reaching consensus among laboratories (Binkely et al.,2004, Sempos et al., 2020).

3.7 Data quality of the present study

We had an arrangement with the reagent manufacturers to deliver the reagents with the same lot numbers. All samples were run in duplicates by a single technician to maintain a constant error.

There were various instruments (Table 3.3) used in the laboratory for the measurements of 25(OH)D, lipid levels and body composition.

Table 3.3: Instruments and techniques used in the laboratory tests

Variables	**Instrument**	**Technique**
25(OH)D (nmol/L)	Diasorin Liaison ®	Chemiluminescence
Lipids [Total Cholesterol, Triglycerides, Low Density Lipoprotein, and High Density Lipoprotein] (mmol/L)	RX Daytona ®	Colorimetry
Body composition (Fat Tissue and Lean Tissue)	DXA	Pencil beam mode technique

From the table above, serum 25(OH)D samples were measured by the Diasorin Liaison instrument which uses the chemiluminescence technique. The lipids (Total cholesterol, triglycerides, High Density Lipoprotein and Low Density Lipoprotein) were analysed by Randox Daytona Instrument which uses colorimetry. The body composition variables were measured by the DXA machine which uses the pencil beam mode technique. The principles of the tests are in appendixes (I – VI).

PART 2: EMPIRICAL CHAPTERS

The statistical analyses will be discussed in each individual results chapter.

CHAPTER 4: Factors influencing the vitamin D status of 10 year old urban South African children.

Published in Public Health Nutrition, 2011;14(2): 334 – 339

CHAPTER 4: Factors influencing the vitamin D status of 10- year-old urban South Africa children.

4.1 Preface – Empirical Paper 1

This chapter was published as an empirical research paper as follows: Poopedi MA, Norris SA, Pettifor JM, Factors influencing the vitamin D status of 10 year old urban South Africa children. Public Health Nutrition, 2011;14(2), 334 – 339.

The manuscript's main contribution to the thesis was to bring to the fore information on vitamin D in 10 year old children (black and white) living in greater Johannesburg metropolitan area and factors influencing their vitamin D status. This was important in addressing the gaps in vitamin D research because of paucity of data on vitamin D status in children. Black children had significantly lower 25(OH)D levels as compared to their white peers. Factors found to influence vitamin D status were season, ethnicity, gender and total fat mass. In overall, 74% of the children had sufficient vitamin D status, it was suggested that vitamin D supplementation or fortification was not necessary in healthy South African children living in Johannesburg.

M.A.P. was responsible for writing the initial drafts of the manuscripts and laboratory running of the blood samples for 25-hydroxyvitamin D and Parathyroid hormone (PTH). S.A.N was the study supervisor for the project and its design; J.M.P. was the study co-supervisor for the project and its design. All authors read and approved the final manuscript as submitted.

4.2 Abstract

Introduction: Hypovitaminosis D is pandemic globally. Lack of data on vitamin D status in children has led to the assessment of vitamin D status in healthy 10-year-old urban children and the factors that influence vitamin D status in these children. **Objective:** Assessment of vitamin D status in a cohort of healthy 10-year-old urban children and the factors that influence vitamin D status in these children. **Design:** A cross-sectional study. Blood samples were collected across four seasons of the year for the biochemical determination of serum 25-hydroxyvitamin D (25(OH)D). Anthropometric measurements (height and weight), BMI and total fat and lean mass (determined by the dual energy X-ray absorptiometry) were measured. 25(OH)D concentrations were assessed by chemiluminescent assay. **Setting:** Study of children in the Greater Johannesburg area of South Africa who form the Bone Health sub-cohort of the longitudinal Birth to Twenty cohort. **Subjects:** Three hundred and eighty-five children who form the Bone Health sub-cohort of the longitudinal Birth to Twenty cohort. **Results:** White children had significantly higher 25(OH)D than their black peers (120.0 ± 36.6) nmol/l versus 93·3 ± 34·0) nmol/l, respectively). Seasonal variations in 25(OH)D levels were found only in white children, with 25(OH)D levels being significantly higher in white than in black children during the autumn and summer months. In multiple regression analysis, season, ethnicity, sex and total fat mass were the factors found to have an influence on 25(OH)D. Vitamin D deficiency (7 %) and insufficiency (19 %) were uncommon among the 10-year-old children. **Conclusions:** Vitamin D supplementation or fortification is not warranted in healthy children living in Johannesburg. However, further studies need to confirm this in other regions of the country, especially in those living further south and with less sunshine during the winter months.

4.3 Introduction

The classical role of vitamin D in humans is to increase the absorption of calcium and phosphate from the gastrointestinal tract (GIT) for the mineralization of the skeleton. A deficiency of vitamin D leads to hypocalcaemia and bone disease (rickets or osteomalacia). Vitamin D has two main forms, vitamin D_3 or cholecalciferol, which is formed in the skin after exposure to ultraviolet light, and ergocalciferol or vitamin D_2 which is obtained by irradiation of ergosterol in plants and foods (Lips, 2006). Serum 25-hydroxyvitamin D [25(OH)D] concentrations serve as a marker of vitamin D status however the optimal levels for health remains a subject of much debate (Holick, 2007). This is attributed to the difficulty in reaching consensus in defining optimal 25(OH)D concentrations not only for bone health at different ages but also for the other non-classical actions of vitamin D on cell differentiation, proliferation and function (Harris, 2006a, Holick, 2008). Some of the uncertainty has also been caused by a lack of standardization of and variation in 25(OH)D assays (Carter et al., 2004).

There are a variety of factors that are reported to influence vitamin D status in children and adults. These include duration of sun exposure, season, cloud cover, latitude, pollution, sunscreen use, skin coverage by clothing, ethnicity and body composition. The paucity of data on the vitamin D status of healthy children in Johannesburg has led us to assess the vitamin D status in healthy 10 year old children and to determine the influence of body composition, seasonal change, sex and ethnicity on the vitamin D status.

Our hypothesis was that children living in Johannesburg would generally be vitamin D sufficient as Johannesburg has greater than 8 sunshine hours daily throughout the year (SACI, 2009); however black children in general would have lower 25(OH)D concentrations than white children due to their greater skin pigmentation.

4.4 Methods

4.4.1 Subjects

This is a cross-sectional study that comprised of 475 subjects (children) aged 10 years, who formed the Bone Health sub-cohort of the Birth to Twenty cohort. Birth to Twenty cohort is a longitudinal study of child health and development, which has followed the development of 3273 children in the Greater Johannesburg area of South Africa since their birth in 1990 (Richter et al., 2004). The Bone Health cohort which is a representative of the larger Birth to Twenty cohort,

consists of 475 black and white children who since the age of 9 years have been intensively studied to investigate factors influencing bone mass accrual during adolescence. The selection of the subjects and cross checks for key demographic variables between the Birth to Twenty and Bone Health Cohort have been reported elsewhere (Vidulich et al., 2006). Participants with chronic illness (juvenile ideopathic arthritis, asthma or epilepsy), on medication which affect growth or bone mass development, or on mineral or vitamin D supplements were excluded. Guardians gave written informed consent for their children to be studied, and verbal assent was obtained from the children. The study protocol was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand, Johannesburg, (Approval number M980810).

All children lived in Johannesburg at a latitude of approximately 26 °S and altitude of 1750 m. Seasons of the year for the statistical analysis of 25(OH)D concentrations were categorized as follows; autumn (March - May), winter (June – August), spring (September – November) and summer (December – February). The average daily sunshine hours in Johannesburg during the seasons are as follows: autumn (8.2 hrs 22^0C), winter (9.1 hrs 18^0C), spring (8.8 hrs 24^0C) and summer (8.2hrs 26^0C)(SACI, 2009). With regard to the sampling procedure of study participants across the seasons, eighty-two participants were sampled in Autumn (seventy-two Blacks and ten Whites), 105 in Winter (seventy-six blacks and twenty-nine whites), 94 in Spring (ninety-one blacks and three whites) and 104 in summer (fifty-six blacks and forty-eight whites), totalling to 385 subjects.(Flow diagram in next page) There were major differences in socio-economic status between the black and white children. Black South African children are exposed to a multitude of factors known to impact negatively on their health in general such as poor nutrition, low dietary calcium intake, less physical activity as well as compromised growth and delayed onset of puberty compared with their white peers (Pettifor et al., 1979, Cameron and Wright, 1990, McVeigh et al., 2004b, MacKeown et al., 2007, McVeigh et al., 2007, Willey et al., 2009)

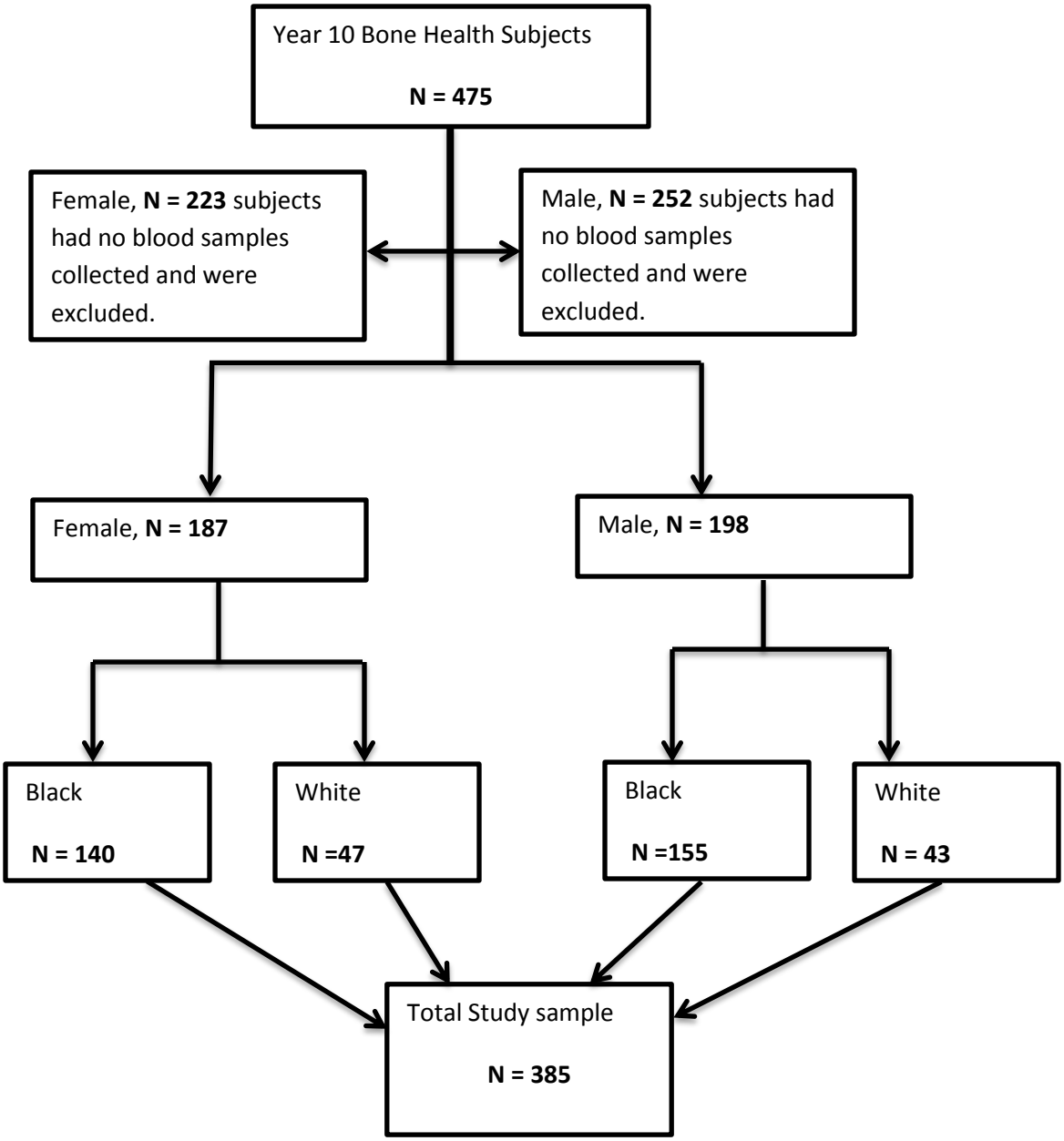


Figure 4.1: Flow diagram of Year 10 Bone Health subjects. Year 10 participants comprised of a total of 475, with the exclusion of 223 females and 252 males due to insufficient or no blood samples to perform laboratory tests. This led to a total sample size of 385 participants (Female, Black-N= 140, White- N= 47 and Male, Black-N = 155, White-N = 43).

4.4.2 Anthropometric measurements and body composition

Height was measured in millimeters (mm) using a wall-mounted stadiometer (Holtain, UK) and weight in kilograms (kg) using a digital electronic instrument (Dismed, USA). Both instruments were regularly calibrated and subjects wore minimal clothing when being weighed. Body mass index (BMI) was calculated as weight (in kg)/height^2 (in m). Total and percentage fat mass and lean mass were measured by DXA, using an Hologic QDR 4500 (Hologic Inc, Bedford, MA, USA). The data were analysed with the software supplied by the manufacturer, version 11.2.

4.4.3 Biochemical analysis

Blood samples (20ml) were drawn by venipuncture from fasting participants into plain tubes by registered nurses. The blood samples were allowed to clot for a minimum of 20 minutes, serum was aliquoted and stored in eppindorf tubes at -70^{o}C until analysed. 25(OH)D was measured by a chemiluminescent assay using DiaSorin Liaison kits (DiaSorin, Stillwater, Minn). All the samples were run in duplicate. Our laboratory is currently participating in the international Vitamin D External Quality Assessment Scheme (DEQAS). Our laboratory was granted the certificate of efficiency, because it has met the performance target set by the DEQAS advisory panel i.e 80% or more of the results fell within ± 30% of the all laboratory trimmed mean (ALTM). The inter-assay coefficient of variation for low and higher 25(OH)D controls was 10% and 9% respectively, while the intra-assay coefficient of variation was 8% and 6% for low and higher 25(OH)D controls respectively. Blood samples were collected from only 385 of the 475 subjects in the cohort as the others refused blood sampling or were not available, or blood samples could not be obtained. As a result 90 subjects (19%) of our study population were without vitamin D data.

The following categories were used to define vitamin D status: vitamin D deficiency (< 50 nmol/L), insufficiency (50 – 74 nmol/L) and sufficiency (≥75 nmol/L) (Holick, 2007, Holick, 2008, Holick, 2009).

4.4.4 Statistical analysis

The results of the present study are expressed as mean ± SD, unless otherwise indicated. The data were analysed using Statistica software version 6 (StatSoft, 2001). Unpaired t-tests were used to compare the means of different groups. All tests were two-tailed and a p-value less than 0.05 was considered statistically significant.

4.5 Results

Blood was available from 385 (140 black female, 47 white female, 155 black male and 43 white male) of the 475 children. Only those subjects with 25(OH)D values were included in the study (Table 1).

Table 4.1: Gender and ethnic differences in age, anthropometry, body composition and 25(OH)D of 10 year old children.

	Female		**Male**		**P-Value**
Variable	Black (N= 140)	White N = 47)	Black (N = 155)	White (N = 43)	P1=Gender Diff P2=Ethnic Diff
Age (years)	10.5 ± 0.3	10.6 ± 0.3	10.5 ± 0.3	10.6 ± 0.3	P1=NS;NS P2=NS;NS
Anthropometry					
Weight (kg)	34.8 ± 8.5(a)	35.9 ± 7.6	32.6 ± 6.4(a)	35.8±6.8	P1=0.01;0.97 P2=NS;NS
Height (mm)	139.2 ± 6.4 (a, b)	143.6 ± 7.3(b)	137.3 ± 6.1 (a,b)	143.2 ± 7.1(b)	P1=0.01;NS P2=0.0001;0.0001
BMI (kg/m2)	17.8 ± 3.4	17.2 ± 2.3	17.2 ± 2.6	17.4 ± 2.5	P1=NS;NS P2=NS;NS
Body Composition Total fat Tissue (g)	10069 ± 5166(a)	9893 ± 4049	7307 ± 3771(a)	8112 ± 3462	P1=0.0001;NS P2=NS;NS
% Fat	28 ± 7.0(a)	27 ± 5.5(a)	22 ± 6.3(a)	22 ± 5.2(a)	P1=0.0001;0.0004 P2=NS;NS
Total Lean Tissue (g)	23892 ± 4009 (a,b)	25164 ± 4020 (a,b)	24152 ± 3151 (a,b)	26825 ± 3849 (a,b)	P1=0.0001;0.03 P2=0.04;0.0001
% Lean Tissue	70 ± 6.8(a)	70 ± 5.3(a)	75 ± 6.0(a)	75 ± 5.0(a)	P1=0.0001;0.0003 P2=NS;NS
Blood Biochemistry Serum-25(OH)D (nmol/l) (Total)	86 ± 31.1(a,b)	112 ± 34.8(a,b)	100 ± 34.4(a,b)	129 ± 37.1(a,b)	P1=0.0004;0.02 P2=0.0001;0.0001

P1= Gender diff [Black (female versus male), and White (female versus male)]

P2 = Ethnic diff [Female (black versus white), and Male (black versus white)]

A P value < 0.05 is considered statistically significant

a = Gender difference, $p < 0.05$

b = Ethnic difference, $p < 0.05$

There was no significant sex or ethnic differences with respect to age between the study groups. Black girls were heavier and taller than black boys (p = 0.01). There were no significant differences in weight, height or BMI between white boys and white girls. White boys and girls were taller than their black (boys and girls) peers (p = 0.0001). There were no significant differences in BMI between any of the groups.

Girls had greater percentage fat and lower percentage lean tissue and total lean tissue than their male peers. There were no significant differences between black and white girls with respect to total fat tissue, % fat or % lean tissue, however, white girls had greater total lean tissue than black girls (p = 0.04). Similarly there were no significant differences between black and white boys with respect to total fat tissue, % fat or % lean tissue, but white boys had greater total lean tissue than black boys (p = 0.0001).

Boys [black (100 ± 34.4 nmol/L) and white (129 ± 37.1 nmol/L)] had significantly higher 25(OH)D than girls [black (86 ± 31.1 nmol/L) and white (112 ± 34.8 nmol/L)] in each ethnic group (p = 0.0004 and 0.02 for black and white children respectively).

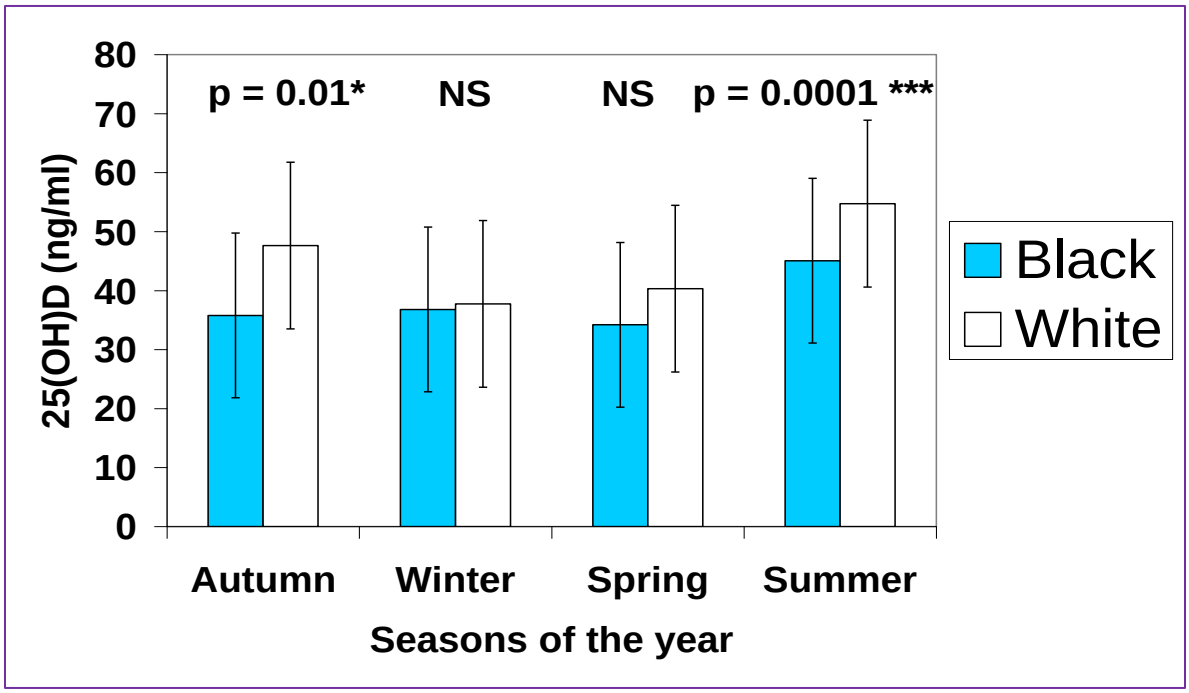


Figure 4.2: 25(OH)D concentrations by season in black and white 10 year old children

Seasonal variations in 25(OH)D were found in the white group of children, with values being highest in summer and autumn. No seasonal variations were noted in the black children.

25(OH)D values were significantly higher in white than black children during the autumn (whites (119 ± 34.1 nmol/L) vs blacks (89 ± 32.3 nmol/L), p = 0.01) and summer months (whites (137 ± 34.9 nmol/L) vs blacks (113 ± 30.9 nmol/L), p = 0.0001) (Figure 1).

The percentages of the black and white subjects with vitamin D sufficiency were 70% and 87% respectively, while 22% and 12% respectively were vitamin D insufficient, deficiency was found in 8% Black and 1% White children. For the overall study population, 7% had vitamin D deficiency, 19% vitamin D insufficiency and 74% vitamin D sufficiency.

In both black and white children 25(OH)D concentrations correlated negatively with percentage fat tissue (r = -0.14, p = 0.02; and r = -0.3, p = 0.01 respectively) and positively with percentage lean tissue, (r = 0.14, p = 0.02 [black], r = 0.27, p = 0.01[White]). After adjusting fat mass and lean mass for height a significant correlation was found between fat mass and 25(OH)D (r = -0.13, p = 0.01).The relationship between 25(OH)D and parathyroid hormone was not significant in either ethnic (Black, r = -1.05, p-value = 0.06 and White, r = -0.09, p-value) or gender groups (Female, r = 0.03, p-value = 0.92 and Male, r = -0.44, p-value = 0.07).

Vitamin status is known to be influenced by variety of factors. Bivariate and multivariate analysis were used to assess factors the influence 25(OH)D levels in Year 10 children.

Table 4.2: The bivariate and multivariate analysis of factors influencing 25(OH)D levels in Year 10 children

Factors versus 25(OH)D	**Gender** β (p-value)	**Ethnicity** β (p-value)	**Season** β (p-value)	**BMI** β (p-value)	**Total Fat (g)** β (p-value)	**% Fat Tissue** β (p-value)	**Total Lean Mass (g)** β (p-value)	**% Lean Tissue** β (p-value)	**Fat Mass Index (FMI)** β (p-value)	**Lean Mass Index (LMI)** β (p-value)	**PTH** β (p-value)
Bivariate Analysis	-0.19	-0.31	-0.18	-0.10	-0.13	-0.83	0.0001	0.86	-23.6	2.79	0.08
	(0.0001)	**(0.0001)**	**(0.0001)**	**(0.0001)**	**(0.0001)**	**(0.02)**	0.96	**0.002**	**0.01**	0.86	0.10
Multivariate Analysis	-0.16	-0.29	0.28	-	-0.15	-2.21	-	-0.68	2.1	-	-
	(0.003)	**(0.0001)**	**(0.0001)**		**(0.01)**	(0.74)		(0.92)	0.07		

Significant p-value < **0.05**

Gender, ethnicity, season, BMI, total fat tissue, percentage fat and FMI were inversely related to 25(OH)D and positively related to percentage lean tissue. When all significant variables from bivariate models were entered into multivariate model for analysis; gender, ethnicity, season and total fat tissue were significantly inversely with associated 25(OH)D levels except for season which was positively associated with 25(OH)D levels.

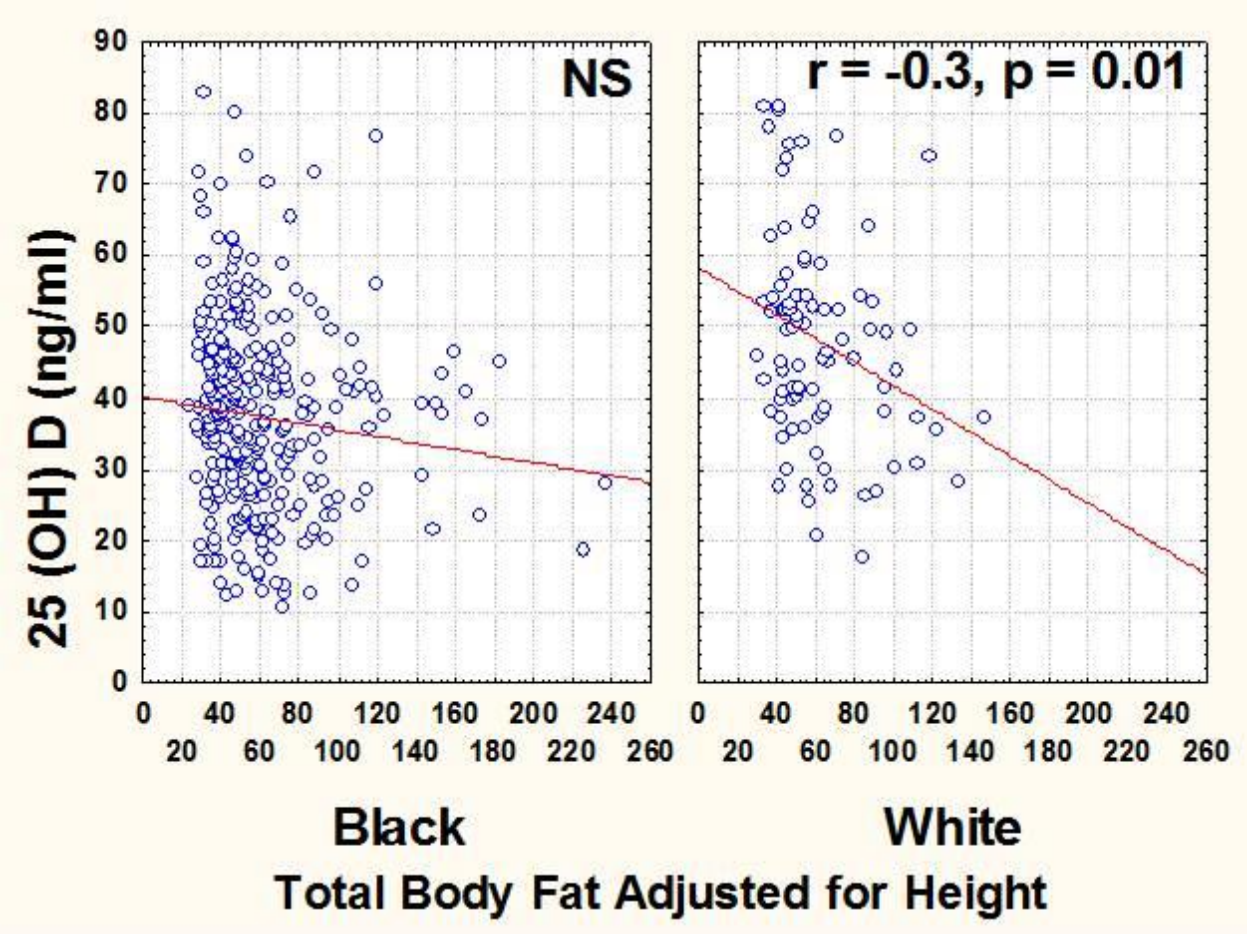


Figure 4.3: Association of 25(OH)D levels with total body fat adjusted for height. The association of 25(OH)D levels with total body fat adjusted for height was found in white children only.

4.6 Discussion

Vitamin D sufficiency was present in 74% of our study population. As we had hypothesized, vitamin D insufficiency is not a major public health issue among 10 year old South African children living in Johannesburg. These findings differ from other studies reported from North Africa (Allali et al., 2009), USA (Harris and Dawson-Hughes, 1998, Harris, 2006b), Europe (Outila et al., 2001), Middle East (Gannage-Yared et al., 2000, El-Hajj et al., 2001), Asia (Agarwal et al., 2002, Harinarayan et al., 2008), New Zealand (Rockell et al., 2005) and elsewhere (El-Sonbaty and Abdul-Ghaffar, 1996). South Africa, and in particular Johannesburg, has abundant sunshine throughout the year (SACI, 2009); thus despite few foods being fortified with vitamin D, vitamin D sufficiency is maintained in healthy ambulatory children. It should be noted however that the majority of children included in the study lived in small separate houses with yards; thus the findings might be very different if children living in high rise apartment blocks in the inner city of Johannesburg had been studied. Factors such as latitude, the lack of excessive clothing coverage (El-Sonbaty and Abdul-Ghaffar, 1996, Ghannam et al., 1999, Gannage-Yared et al., 2000, Mishal, 2001) and not living in high-rise urban inner city ghettos may have played significant roles in reducing hypovitaminosis D in the present study.

As in other studies, ethnic differences in 25(OH)D levels were found in the present study. White children had significantly higher 25OHD than their black peers (Table 1.). Dark skin colour of black participants is known to restrict the solar UVB photons that penetrate the skin thereby reducing the cutaneous production of vitamin D_3 (Clemens et al., 1982). Numerous studies comparing black and white young and old subjects in different countries have reported that blacks have lower 25(OH)D levels than whites (Harris, 2006b, Stein et al., 2006). It is a well-known fact that pigmentation reduces vitamin D production in the skin. However, studies (Clemens et al., 1982, Lo et al., 1986) have shown that darker skin subjects have the capacity to produce vitamin D of similar amount to light skin subjects when exposed to prolonged UVB sunlight. In our study, the lower 25(OH)D in black children during summer probably reflects this difference in the capacity to produce vitamin D with similar exposures.

Dietary calcium intake in this cohort of children at the age of 9 years was significantly lower in blacks of both sexes (347 mg/d), compared with white female (719 mg/d) and male participants (778 mg/d) (McVeigh et al., 2004a). Low calcium intake in black children could result in increased catabolism of 25(OH)D and thus accentuate the lower 25(OH)D. We did not find a

relationship between 25(OH)D and PTH in either ethnic or sex groups, supporting the contention that the majority of children were vitamin D replete.

One of the factors that influence vitamin D status in both young and adult subjects is season. Seasonal changes in 25(OH)D levels have been reported in black and white subjects and other ethnic groups (Dawson-Hughes et al., 1997, Rockell et al., 2005). In the present study, the effect of season on serum 25(OH)D levels was only seen in the white children. The biggest difference in 25(OH)D concentrations between black and white children was noticed during autumn and summer ($p = 0.01$ and 0.0001), while there was no significant ethnic difference in 25(OH)D in winter and spring (Figure 1). The reason for the lack of seasonal variation in black children is not clear, but it is interesting to speculate that black children do not spend time lying in the sun at swimming pools during the summer months because of the differences in socioeconomic status and the lack of facilities in the mainly black communities. This finding of a lack of seasonal variation in black children is different to that reported in some studies of children (Looker et al., 2002, Rockell et al., 2005) but similar to others (Stein et al., 2006). The present study was conducted at a latitude of approximately 26°S and an altitude of 1750m, and also evidence suggest that there is a constant vitamin D production in Johannesburg, with abundance of sunlight all year round. This probably contributed to the sufficient vitamin D status of our study subjects.

Interestingly, the association of 25(OH)D levels with total body fat adjusted for height was observed in white children and not their black peers. Hence it was suggested that the black and white children had different body fat distribution. In a multiple regression analysis, season ($\beta = 0.28$, $p = 0.0001$), ethnicity ($\beta = -0.29$, $p = 0.0001$), sex ($\beta = -0.16$, $p = 0.003$), and total fat mass ($\beta = -0.15$, $p = 0.01$) were the only factors found to have an influence on 25(OH)D. Higher 25(OH)D levels were found in male subjects than female subjects in the present study, a finding which is similar to other studies (Rockell et al., 2005, Dawson-Hughes et al., 1997). The gender difference in our present study may reflect the fact that males may spend more time outdoors (but this was not measured). A study of physical activity in the same group of children has previously revealed that males have higher physical activity than their female peers in both ethnic groups (McVeigh et al., 2004b, McVeigh et al., 2007). Some researchers have suggested that gender differences in vitamin D status may be linked to androgen-related differences in vitamin D binding protein levels, to differences in precursor production in the skin, to differences

in 25-hydroxylation by the liver (Carnevale et al., 2001), or to gender differences in body fat. The girls in our study had significantly greater fat mass than the boys, thus possibly providing another reason for the sex differences in 25(OH)D concentrations in our cohort. Vitamin D is a fat-soluble vitamin, and the inverse relationship between 25(OH)D and fat mass has been reported previously by researchers (Wortsman et al., 2000, Arunabh et al., 2003). This association has been attributed to the sequestration into adipocytes of vitamin D generated in the skin or orally ingested, before it can be transported to the liver and converted to 25(OH)D (Wortsman et al., 2000, Arunabh et al., 2003). However, it still remains unclear whether adiposity (or percentage body fat) should be taken into consideration while assessing vitamin D requirements in the general population. We found a similar inverse relationship between fat mass and 25(OH)D in our black and white children.

In conclusion despite seasonal variations in 25(OH)D levels, vitamin D deficiency [25(OH)D < 50 nmol/L] and vitamin D insufficiency [25(OH)D 50 – 74 nmol/L] were uncommon findings in our black and white 10 year old children. We therefore believe that vitamin D supplementation or fortification should not be considered in healthy South African children living in Johannesburg. Whether similar findings hold true in other regions of the country need to be confirmed, however of particular interest would be studies in Cape Town, where a previous study has shown only limited vitamin D synthesis *in vitro* from April through to September (Pettifor et al., 1996).

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CHAPTER 5: Does Vitamin D track through adolescence?

Published in: *American Journal of Clinical Nutrition 2015; 102(5): 1025 – 1029.*

Chapter 5: Does Vitamin D track through adolescence?

5.1 Preface – Empirical Paper 2

This chapter was published as an empirical research paper as follows: Poopedi MA, Norris SA, Micklesfield LK, Pettifor JM, Does Vitamin D track through adolescents? American Journal of Clinical Nutrition 2015, 102, 1025 – 1029.

The manuscript's present the second part of the study by investigating vitamin D tracking in adolescent children over a period of 10 years. This is because majority of studies have assessed vitamin D status by a single measurement of 25 (OH) D in both young and elderly only once. On the other hand longitudinal assessment of vitamin D was published by few researchers with contradicting results. In the present study, there was evidence of vitamin D tracking over a shorter period of time (year 11 and 13, and year 15, 17 and 20). There were nearly 50% of participants who had a change in 25(OH)D of 20 nmol/l or greater between 11 and 20 years of age. Only one third of adolescents were vitamin D replete at both 11 and 20 years of age. However, 20% of the cohort who were vitamin D insufficient at 11 years of age, were replete at 20 years. The present study has shown that a single measurement may not accurately reflect the vitamin D status of an individual over a prolonged period of time.

M.A.P. was responsible for laboratory running of the blood samples for 25-hydroxyvitamin D and writing the initial drafts of the manuscripts; S.A.N and JMP were the study supervisors for the project, conceptualised and design the study and edited the drafts; J.M.P. was the study co-supervisor for the project and its design. KLM assisted with the statistics and reviewed the drafts. All authors read and approved the final manuscript as submitted.

5.2 Abstract

Introduction: To our knowledge, no studies have reported on the long-term variability of vitamin D status in adolescents. **Objective:** To determine whether tracking of vitamin D status occurs in healthy adolescents, we assessed the variability of 25-hydroxyvitamin D (25(OH)D) every 2 years over a 10 year period in a longitudinal cohort of adolescents living in Johannesburg, South Africa (latitude 26°S). **Methods:** Healthy adolescents who had blood samples available on three or more occasions between 11 and 20 years of age were included in the study. Of the cohort of 504 children, 99 met the criteria. The mean 25(OH)D for each time point was determined, as well as the individual 25(OH)D z-scores based on year 11 values as the reference. All the 25(OH)D measurements for a subject were measured in a single assay. **Results:** No significant correlation between 25(OH)D in the earlier and later years of adolescence, although there were significant correlations between year 11 and year 13 ($r = 0.71$; $p < 0.0001$), and between years 15, 17 and 20 ($r \geq 0.65$; $p < 0.0001$). The percentage of adolescents whose 25(OH)D concentration changed by > 20 nmol/L from year 11 was calculated for all age groups: 12% of the cohort had a change of > 20nmol/L at 13 years of age compared to 46% at 20 y of age. Just more than one-half (53%) of the cohort changed their category of vitamin D status between the ages of 11 and 20 y, one-third of adolescents changed from being replete to insufficient over the same period. **Conclusion:** The data suggest that the measurement of 25(OH)D at a single time point does not reflect the long-term vitamin D status of an adolescent. These findings may throw doubt on the veracity of those studies suggesting associations of vitamin D status with various disease states in which vitamin D status was only measured once.

5.3 Introduction

The classic role of vitamin D in calcium and bone homeostasis, as well as its role in cell differentiation, function and proliferation is well known (Holick, 2004). Recently, attention has focused on the high prevalence of low vitamin D status in many communities around the world (Mithal et al., 2009, Lips, 2010), including countries both at low and high latitudes (Durazo-Arvizu et al., 2014). Many studies have reported associations between vitamin D status and the prevalence of diseases such as asthma, autoimmune diseases, and some cancers, yet in the majority of these studies the vitamin D status has been assessed by a single measurement of 25-hydroxyvitamin D (25(OH)D) (Holick, 2004, Bose et al., 2013, Montero-Arias et al., 2013, Bikle and Jiang, 2013). It is possible that the values obtained may not reflect the individuals' vitamin D status over time, as they may vary as a consequence of changes in the factors which influence vitamin D intake, or formation in the skin (el-Sonbaty and Abdul-Ghaffar, 1996, Agarwal et al., 2002, Allali et al., 2009). Few studies have assessed the change in vitamin D status in participants over time. In a Norwegian study, in which 25(OH)D was measured 18 years apart in over 2700 adults, the correlation between the two 25(OH)D z-scores was 0.42, and approximately 46% of subjects had a < 10 nmol/L change in 25(OH)D over the 18 year (Jorde et al., 2010). In further analysis, the authors selected 94 adults from the initial sample, who had 25(OH)D levels measured every three months for one year, and showed that approximately 71% had a change of 25(OH)D of < 10 nmol/L from baseline and 95% had a change in 25(OH)D of < 20 nmol/L at the end of the tracking. A Canadian study that examined vitamin D status over a period of 10 years reported that approximately 50% of subjects had a change in 25(OH)D levels > 20 nmol/L over the 10-year period (Berger et al., 2012).

Studies on vitamin D status in South African populations have been conducted in adults and all studies tested 25(OH)D levels at one time point (Daniels et al., 1997, Kruger et al., 2011, Martineau et al., 2011, George et al., 2014a). In South Africa, there is limited vitamin D intake from the diet as few foods are vitamin D fortified, and thus the population is almost totally dependent on skin synthesis of vitamin D. The use of vitamin D supplements is uncommon in the majority of the population (Poopedi et al., 2011, George et al., 2014b). During adolescence, major changes generally occur in an individual's lifestyle, with possible reductions in physical activity and the time spent outdoors, and increases in skin coverage by

clothing as the adolescents grow older (Kimm et al., 2002, Kampman et al., 2007). Thus one might expect that during this period there would be major changes in 25(OH)D concentrations during this period, however few data exist on vitamin D status in this age group and, to our knowledge, no studies have reported on the long-term variability of vitamin D status. Therefore, we prospectively studied the variation in serum 25(OH)D levels over a 10 year period in a longitudinal cohort of adolescents living in Johannesburg, South Africa.

5.4 Methods

5.4.1 Subjects

The subjects were healthy urban South African children living in the greater Johannesburg Metropolitan region (latitude 26°S), who formed part of the Bone Health sub-cohort of the longitudinal Birth to Twenty cohort. The Birth to Twenty cohort's characteristics, recruitment, and exclusion and inclusion criteria have been reported in previous publications (McVeigh et al., 2004, Vidulich et al., 2006, Poopedi et al., 2011). Briefly, this cohort of children has been followed since their birth in 1990 and consisted initially of 3273 black and white singleton deliveries that occurred within the Greater Johannesburg Metropolitan area over a 6-week period. The Bone Health cohort, which is representative of the larger Birth to Twenty cohort, consists of 504 black and white children who, since the age of 9 years, have been intensively studied annually to investigate factors influencing bone mass accrual and fracture risk during adolescence. Children returned for annual visits as close to the birth month as possible over a period of 10 years. The study protocol was approved by the Committee for Research on Human subjects of the University of the Witwatersrand, Johannesburg (Approval no. M980810), and a parent/guardian gave written informed consent for their children to participate in the study, and participating adolescents gave verbal assent or written consent depending on whether or not they were younger than 18 years of age.

5.4.2 Selection criteria

Children with metabolic bone diseases or chronic illnesses such as juvenile rheumatoid arthritis, epilepsy or asthma, or who were on vitamin D supplements or medications that affect bone mass or calcium homeostasis were excluded from the study. Serum 25(OH)D was measured biennially over the 10 year period from 11 to 20 years of age. Only those children

who had serum available for the measurement of 25(OH)D on ≥ 3 occasions over this period were included. Of the initial 504 children, 99 met these criteria. Below is the flow diagram.

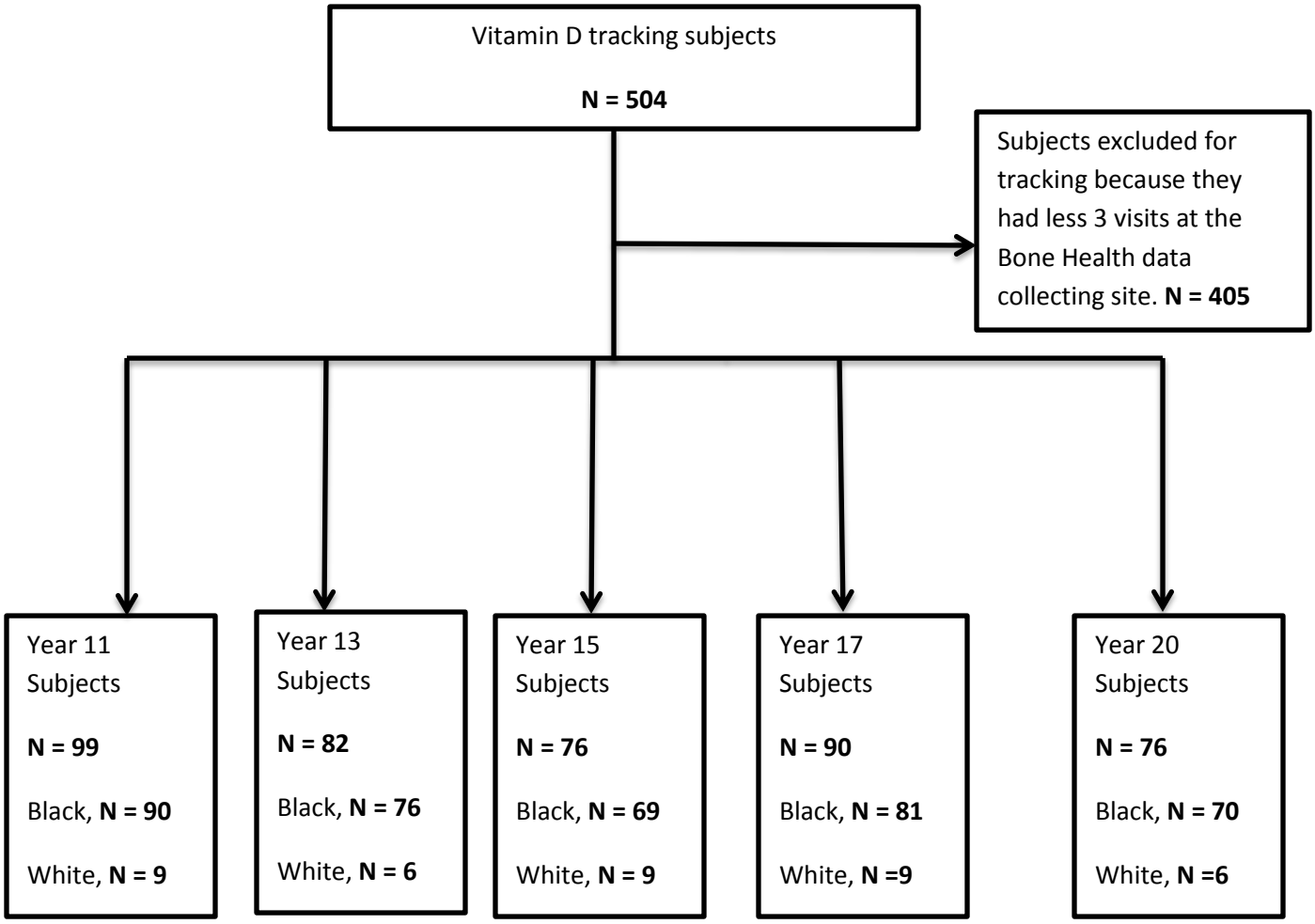

Figure 5.1: Flow diagram for vitamin D tracking subjects. At the beginning of the study, 504 participants were eligible to be part of vitamin D tracking. Subjects (N = 405) were excluded from vitamin D tracking because they had less than 3 visits at the Bone Health data collection site.

5.4.3 Measurement of serum 25(OH)D

Fasting serum samples after separation were analyzed for 25(OH)D by chemiluminescent assay (Diasorin Liason, Stillwater, MN, USA) in a laboratory which participates in an international quality assurance programme (DEQAS - International Vitamin D External Quality Assessment Scheme, London, United Kingdom). Since our participation in DEQAS external quality control, our laboratory had received the certificates of efficiency on yearly basis (i.e 80% or more results fell within 30% of the all laboratory trimmed mean). The same

control and reagent lots were used for the analysis of the samples and all the samples, in duplicate, from an individual participant were run in the same assay. The coefficients for inter-assay variation for low and high controls were 8% and 6% respectively and for intra-assay variation for low and high controls were 5% and 3% respectively.

5.4.4 Statistical analysis

The data were analyzed using the Statistica software package version 6 (StatSoft, Tulsa, OK, USA). Age, height, weight, BMI and 25(OH)D concentrations were normally distributed. In order to assess tracking patterns, 25(OH)D z-scores were calculated for each participant at each time point using the mean and standard deviation values of year 11 as the reference value. The changes in 25(OH)D z-scores and actual 25(OH)D levels between baseline and the other years were calculated by subtracting the relevant values of the particular year from the baseline (Year 11) z-scores or actual concentrations. One way ANOVA (analysis of variance) tests were used for the analysis of differences in mean height, weight, BMI, 25(OH)D levels and their respective mean z-scores among the five age groups, and a post hoc test (least significant difference) was used to determine individual group differences. A p-value of < 0.05 was considered as statistically significant. Pearson correlation tests were conducted to determine association between 25(OH)D values measured at the various time points. The change in 25(OH)D between time points in each subject was divided into 3 categories of < 10 nmol/L, 10 – 20 nmol/L and > 20 nmol/L, in order to assess the 25(OH)D stability over a period of 10 years.

5.5: Results

Table 5.1. Mean 25(OH)D concentrations and z-scores at five time points during adolescence

Variable	Year 11 (N = **99**) Baseline Black (N=90) White (N= 9)	Year 13 (N = **82**) Black (N=76) White (N=6)	Year 15 (N = **76**) Black (N=69) White (N=9)	Year 17 (N = **90**) Black (N=81) White(N= 9)	Year 20 (N = **76**) Black (N=70) White(N= 6)	ANOVA P-Value
Age (Years)	11.6 ± 0.3	13.7 ± 0.2	15.6 ± 0.3	17.7 ± 0.2	20.8 ± 0.6	
Height (cm)	143 ± 7.3	155 ± 8.1	162 ± 8.7	165 ± 8.8	167 ± 7.9	**<0.0001****
Height Z-Scores	-0.004 ± 0.10	-0.01 ± 0.10	-0.003±0.10	0.001 ± 0.10	0.01 ± 0.10	1.00(NS)
Weight (kg)	37 ± 8.4	48 ± 11.2	57 ± 12.1	60 ± 12.2	64 ± 14.6	**<0.0001****
Weight Z-Scores	-0.001 ± 0.10	-0.0004 ± 1.0	-0.003 ± 0.10	0.001 ± 0.10	0.003 ± 0.10	0.99 (NS)
BMI (kg/m^2)	18 ± 2.9	20 ± 3.6	22 ± 4.1	22 ± 4.1	23 ± 4.7	**<0.0001****
BMI Z-Scores	-0.02 ± 1.0	-0.001 ± 1.0	-0.005 ± 1.0	-0.004 ± 1.0	-0.01 ± 1.0	0.99 (NS)
25(OH)D (nmol/L)	58 ± 5.8*	58 ± 7.7*	55 ± 7.7	60 ± 7.7*	50 ± 7.2	**< 0.01****
25(OH)D z-scores	0.0001 ± 1.0*	0.05 ± 1.3*	-0.09 ± 1.3	0.19 ± 1.3*	-0.44 ± 1.2	**< 0.01****

*Significant difference between year 20 and other years (actual 25(OH)D values and 25(OH)D z-scores) determined by post-hoc test.

**Significant variations in mean height, weight, BMI, 25(OH)D and 25(OH)D z-scores by age determined by ANOVA test.

***25(OH)D Deficiency; < 30 nmol/L, insufficiency; 30 – 50 nmol/L; > 50 nmol/L

Mean age, height, weight, BMI, and 25(OH)D concentrations and the respective z-scores for the five measurement years are presented in Table 5.1, with the number of subjects varying from 99 at baseline (year 11, males-58% and female-42%) to 76 at the end of the study (year 20, males-59% and females-41%). Significant differences in mean height, weight, BMI, 25(OH)D values and z-scores between the respective age groups were shown by one way ANOVA, (p-value < 0.01). Combining the measurements over all the years, (year 11 to 20; N = 423), 5% of the measurements were deficient (< 30 nmol/L), 35% insufficient (30 – 50 nmol/L) and 60% vitamin

D replete (> 50 nmol/L). Black children had significantly lower 25(OH)D concentrations than their white peers in early (Year 11 - 13) and late (Year 15 - 20) adolescence. However within each ethnic group, mean 25(OH)D was similar in early and late adolescence. The proportion of visits of each individual over the 10 year study period that fell within the same season was approximately 76%.

Seasonal variations in 25(OH)D levels, which were more marked in white than black adolescents (summer/autumn 73 and 57 nmol/L; winter/spring 61 and 53 nmol/L in whites and blacks respectively).

In further analysing the difference between the age groups, post hoc test (least significant difference) was used. The 25(OH)D values and z-scores at year 20 were significantly lower than at year 11 ($p < 0.02$), year 13 ($p < 0.01$), and year 17 ($p < 0.001$) values, but not when compared to year 15.

Table 5.2: Pearson correlation coefficients (r) between individual participant's 25(OH)D (nmol/L) values obtained in each of the study years.

	Year 11 (N = 99)	**Year 13 (N = 82)**	**Year 15 (N = 76)**	**Year 17 (N = 90)**	**Year 20 (N = 76)**
Year 11 (N = 99)		**r = 0.706 p < 0.0001**	r = -0.087 p = 0.6	r = -0.052 p = 0.7	r = -0.151 p = 0.4
Year 13 (N = 82)	**r = 0.706 p < 0.0001**		r = 0.079 P = 0.6	r = 0.011 p = 0.1	r = -0.004 p = 0.8
Year 15 (N = 76)	r = -0.087 p = 0.6	r = 0.079 P = 0.6		**r = 0.733 p = 0.0001**	**r = 0.649 p < 0.0001**
Year 17 (N = 90)	r = -0.052 p = 0.7	r = 0.011 p = 0.1	**r = 0.733 p < 0.0001**		**r = 0.701 p < 0.0001**
Year 20 (N = 76)	r = -0.151 p = 0.3	r = -0.004 p = 0.9	**r = 0.649 p < 0.0001**	**r = 0.701 p < 0.0001**	

The correlations between 25(OH)D values collected at the different times are presented in **Table 5.2:** Year 11 25(OH)D levels correlated significantly with those of year 13 ($p < 0.0001$), but did not correlate with any of the other ages. Years 15, 17 and 20 correlated significantly with each other (all $p< 0.0001$), but not with the earlier years.

A multiple regression analysis (Table 5.3) was performed to determine whether covariates such as gender, ethnicity, seasons of the year, BMI or pubertal status had an effect on 25(OH)D, but no association was found.

Table 5.3: Multivariate analysis for factors known to influence 25(OH)D levels

Years	Gender β (p-value)	Ethnicity β (p-value)	Seasons of the year β (p-value)	BMI β (p-value)	Puberty Status β (p-value)
Year 11 Z-Scores 25(OH)D	0.17 (0.05)	0.49 (0.17)	0.44 (0.37)	-0.07 0.45	**Genital Breast Development** 0.01 (0.96)
Year 13 Z-Scores 25(OH)D	0.15 (0.10)	1.76 (0.09)	0.19 (0.95)	-0.06 (0.13)	**Genital Breast Development** -0.23 (0.49)
Year 15 Z-Scores 25(OH)D	-0.02 (0.95)	0.03 (0.55)	0.23 (0.47)	0.003 (0.93)	**Public hair Breast Development** -0.34 0.44 (0.38) (0.25)
Year 17 Z-Scores 25(OH)D	-0.27 (0.38)	1.18 (0.23)	0.13 (0.68)	-0.028 (0.47)	**Pubic hair Breast Development** -0.49 -0.17 (0.27) (0.68)
Year 20 Z-Scores 25(OH)D	-0.65 (0.37)	0.45 (0.52)	1.05 (0.45)	-0.008 (0.78)	**Breast Development** -1.02 (0.06)

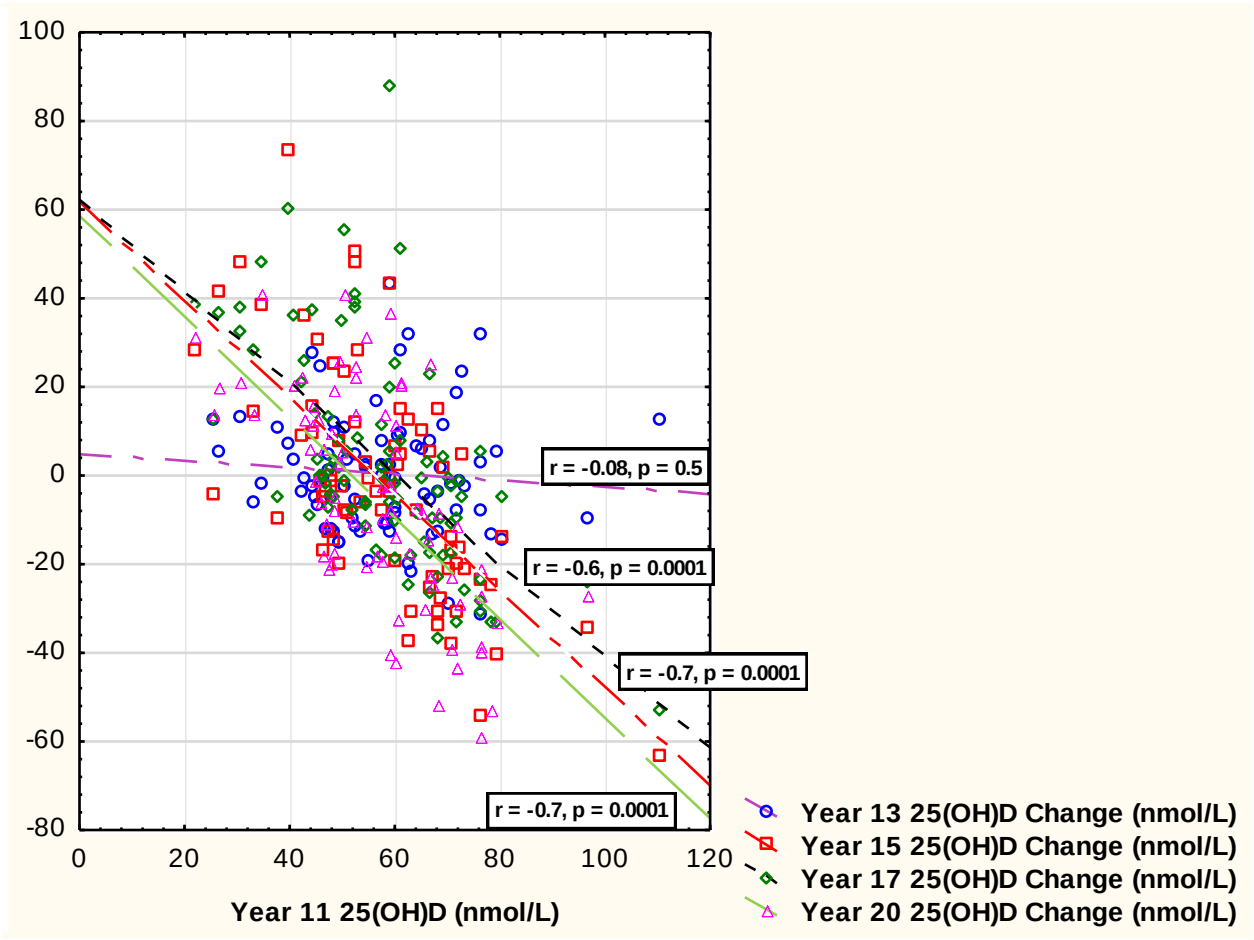

Figure 5.2: Change in 25(OH)D (nmol/L) in subjects over the 10 years of study from the values at age 11 years.

In **Figure 5.1**, the associations between 25(OH)D at age 11 years and changes in 25(OH)D between year 11 and the other ages are presented. There was no significant association between 25(OH)D in year 11 and the change in 25(OH)D between year 11 and year 13, however 25(OH)D concentrations in year 11 were inversely associated with the changes in concentrations between year 11 and all the other years.

Table 5.4: Number (percentage) of subjects with a change in 25(OH)D ≤ 10 nmol/L, 11 – 20 nmol/L or > 20 nmol/L between baseline (11 years of age) and the other time points

Variable	≤ 10 nmol/L	11 – 20 nmol/L	> 20 nmol/L
Year 13 change from year 11 (N = 82)	47 (57 %)	25 (31%)	10 (12%)
Year 15 change from year 11 (N = 76)	28 (37%)	17 (22%)	31 (41%)
Year 17 change from year 11 (N = 90)	15 (17%)	41 (46%)	34 (38%)
Year 20 change from year 11 (N = 76)	18 (24%)	23 (30%)	35 (46%)

The percentage of adolescents who had changes in 25(OH)D concentrations of < 10 nmol/L, 11-20 nmol/L or > 20 nmol/L from baseline to each time point, are presented in **Table 5.3**. Just over half (57%) of year 13 25(OH)D values changed by ≤ 10 nmol/L from year 11, with only 12% of the cohort having a change in value of more than 20 nmol/L over these two ages. However, at the other time points approximately 40% of the cohort had a change of > 20 nmol/L.

Table 5.5: A comparison of the vitamin D status at baseline (year 11) and in the last year (year 20) of measurement

Vitamin D status category	Sample, %
Vitamin D insufficient at year 11 and at year 20	17
Vitamin D replete at year 11 and year 20	30
Vitamin D insufficient at year 11 and vitamin D replete at year 20	20
Vitamin D replete at year 11 and vitamin D insufficient at year 20	33

The percentages of adolescents who maintained or changed their vitamin D status between year 11 and year 20 are shown in **Table 5.4**. Just over half (53%) of the cohort changed their vitamin D status over the two time points, with 33% of the adolescents changing from a replete to an insufficient status over the 10 year period. Just under a third (30%) of adolescents remained vitamin D replete between year 11 and year 20.

5.6: Discussion

The period of adolescence is generally associated with changes in lifestyle, often associated with a reduction in out-doors physical activity, due to the tendency of playing indoors with computers or watching television (Kimm et al., 2002, Kampman et al., 2007). These changes predispose this age group to reduced cutaneous synthesis of vitamin D and thus to alterations in circulating 25(OH)D concentrations, as has been noted in the current study. Another notable thing in the study was that factors (gender, ethnicity, season of the year, BMI and puberty) known to influence 25(0H)D levels were not statistically significant. However, they were statistical significant when the study group was 10 year old. The reason for that is not known but we can speculate that it might be due to loss of follow up in study participants.

Studies in adolescence and adults have reported an association between vitamin D status and various disease states but 25(OH)D levels were measured at one time point only (Mithal et al., 2009, Colao et al., 2014, Modan-Moses et al., 2014). For example, in a retrospective study by Kao et al., 2015 vitamin D deficiency was found to be associated with high blood pressure in obese children, yet the vitamin D status was only measured at the time of assessment of the blood pressure. In another case-control study low serum 25(OH)D appeared to be related to asthma in children, but the conclusions were made based on a single 25(OH)D measurement at the time of assessment (Hatami et al., 2014). As vitamin D status is influenced by factors which affect skin production of vitamin D (Harris and Dawson-Hughes, 1998, Agarwal et al., 2002, Harris, 2006, Shoben et al., 2011), as well as dietary patterns (Holick, 2008) and body composition (Wortsman et al., 2000, Arunabh et al., 2003), we hypothesized that a single measurement of 25(OH)D in healthy adolescent children might not reflect vitamin D status over a prolonged period of time. In this study, we have shown that nearly 50% of participants had a change in 25(OH)D of 20 nmol/L or greater between 11 and 20 years of age. Furthermore, only one third of adolescents were vitamin D replete at both 11 years of age and at 20 years of age. From a positive perspective, 20% of the cohort who were vitamin D insufficient at 11 years of age, were replete at 20 years.

We found that adolescents who had the highest 25(OH)D concentrations at 11 years of age had the largest decrease in 25(OH)D at 15, 17 and 20 years of age, while those with low baseline values had a greater increase at the other time points, suggesting that over time there is a tendency for 25(OH)D concentrations to regress towards a mean.

Although an individual's serum 25(OH)D levels correlated over relatively short periods of time in early and late adolescence, there was no significant correlation over a longer period, thus individual participant levels in years 11 and 13 were not correlated with those in years 15, 17 or 20.

Data on vitamin D tracking is still lacking, with only two studies in adults published with conflicting results. In one study tracking of vitamin D over a short period (12 months) was shown by strong positive correlations between months of tracking (Jorde et al., 2010), whereas there was poor 25(OH)D tracking over a long period (between 5 and 10 years) in other study (Berger et al., 2012). In the present study, tracking was present in early and late adolescence, but not throughout adolescence. We could find no good explanation for this dichotomy in results between early and late adolescence, but it is unlikely to be due to laboratory variations as all samples from a single participant were measured in the same assay. We do acknowledge that only a relatively small proportion of the cohort (99 of 504 participants) were eligible for enrolment into the study due to the requirement of having available blood samples on three different occasions over the study period, which could have introduced bias. To better understand the association between vitamin D status and disease states, more longitudinal studies are required to document the pattern of vitamin D tracking in the participants, and the factors that influence it. It is possible that tracking of vitamin D status is least apparent during periods of major lifestyle changes as occurs in adolescence, or in regions where seasonal variations are large.

Conclusion: In our study of South African adolescents there was no association between 25(OH)D values measured in early and later adolescence. Furthermore nearly 50% of participants had a change in 25(OH)D of greater than 20 nmol/L over the 10 years of study. Thus tracking was restricted to relatively short periods at the beginning and end of adolescence. The present study suggests that a single measurement of 25(OH)D may not accurately reflect the vitamin D status of an individual over a prolonged period of time. These results cast doubt on the veracity of reports that suggest that vitamin D status (as measured on a single occasion) may influence long term disease outcomes.

Acknowledgements

The contribution of Bone Health and Birth to Twenty staff members presently Developmental Pathways for Health Research Unit (DPHRU) in data collection both past and present is greatly appreciated by the authors.

The authors' responsibilities were as follows – MAP: was responsible for running the blood samples for 25(OH)D and writing the initial drafts of the manuscript; JMP and SAN: supervisors of the project; SAN and JMP conceptualised and designed the study and reviewed and edited the drafts. LKM: assisted with the statistics and reviewed the drafts; and all authors: approved the final version of the manuscript. The authors have no conflict of interest to declare.

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CHAPTER 6: Is vitamin D status associated with non-communicable disease risk in children? A cohort study

Under review: South African Journal of Clinical Nutrition

CHAPTER 6: Is vitamin D status associated with non-communicable disease risk in children? A cohort study

6.1 Preface – Empirical Paper 3

This chapter was submitted to the *South African Journal of Nutrition,* where it is still under review. It was submitted as a third empirical research paper as follows: Poopedi MA, Norris SA, Pettifor JM, **Is vitamin D status associated with non-communicable disease risk in children? A cohort study. (Under review; South African Journal of Clinical Nutrition)**

The manuscript's third part of the thesis assessed vitamin D association with non-communicable disease risk (blood pressure, BMI and lipid levels) in children. There was a significant increase in non-communicable disease risk at different time points over a period of 10 years, with a significant decrease in 25(OH)D. However, there was 77% of 25(OH)D replete in the study population. The association of 25(OH)D with non-communicable disease risk was conducted by Generalized Estimating Equations (GEE) to accommodate missing values. There was evidence of variations in 25(OH)D, BMI and BP in adolescents over a period of 10 years. After controlling for covariates, increased 25(OH)D was significantly negatively associated with LDL-C, which may be related to favourable lipid profile in children and adolescents.

M.A.P. was responsible for laboratory running of the blood samples for 25-hydroxyvitamin D, lipid profile (Total Cholesterol (TC), Triglycerides (Trig), High density Lipoprotein (HDL-C) and Low Density Lipoprotein (LDL-C) and writing the initial drafts of the manuscripts; S.A.N and J.M.P were the study supervisors for the project, and conceptualised and designed the study and edited the drafts; J.M.P. was the study co-supervisor for the project and its design. All authors read and approved the final manuscript as submitted.

6.2 Abstract

Background: Studies in children and adults have reported variations in 25-hydroxyvitamin D (25(OH)D), body mass index (BMI), and blood pressure (BP) over time. Furthermore, there has been a reported association of 25(OH)D with BMI, BP and lipid levels in some cross-sectional and longitudinal studies. **Methods:** This is a longitudinal study of a group of adolescents with repeated measures of variables (25(OH)D, BP, anthropometry, and lipids) at the ages of 11, 12 13, 15 and 18-20 years. For age related changes, year 12 participants (N = 261) were matched with year 18-20 (N = 368) participants resulting in 200 in each group. The data for longitudinal analyses using the GEE comprised of the following groups of subjects: Year 11 (N = 288), Year 12 (N = 253), Year 13 (N = 292), Year 15 (N = 238) and Year 18-20 (N = 368). The relationship of 25(OH)D with BMI, BP and lipid levels over a period of 10 years was assessed. **Results:** There were significant increases of mean BMI and BP, and decreases in 25(OH)D levels with age (all p-values < 0.0001). In females, systolic BP was significantly higher in older participants (18-20 years) than younger participants (12 years), but 25(OH)D was significantly higher in younger than older participants. In males, there was significant increase in BP in participants between age 12 years and 18-20 years. 25(OH)D, total cholesterol (TC), and low density lipoprotein (LDL-C) were significantly lower in 18-20 year-old participants compared to 12-year-old participants. Longitudinally 25(OH)D was inversely associated with LDL-C. **Conclusion:** There is evidence of variations in 25(OH)D, BMI and BP in adolescents over a period of 10 years. After controlling for covariates, LDL-C was significantly negatively associated with 25(OH)D, which suggests that vitamin D status might positively be associated with favourable lipid profiles in children and adolescents.

6.3 Introduction

The classical function of vitamin D is to facilitate the absorption of calcium from the gastrointestinal tract (GIT) to maintain serum calcium homeostasis (Kanis, 1994, Harkness and Bonny, 2005). However, recent evidence suggests that vitamin D status may be associated with a variety of non-communicable disease (NCD) risks such as hypertension, obesity, metabolic syndrome and diabetes mellitus (Chiu et al., 2004, Forman et al., 2007, van der Mei et al., 2007, Chacko et al., 2011, Sacheck J, 20011, Pavlovic D, 2011, Yin X, 2016). The mechanisms by which hypovitaminosis D might be related to these diseases remain unclear, although vitamin D may influence immunomodulation, insulin release and cardiovascular function (Fischer et al., 2000, Holick, 2008, Bikle and Jiang, 2013). Vitamin D status is dependent on both dietary intake and cutaneous synthesis through exposure to ultraviolet β radiation, the latter being affected by factors such as latitude, season, skin pigmentation, lifestyle and cultural practices (Clemens et al., 1982, Binkley et al., 2004, Allali et al., 2009, Arunabh et al., 2003a, Bassil et al., 2013). Studies in children and adults have found obesity and sedentary lifestyles to be associated with reduced vitamin D status (Gannage-Yared et al., 2000, Arunabh et al., 2003a). An abnormal lipid profile and high BP have also been associated with vitamin D deficiency (Forman et al., 2007, Rajakumar et al., 2011a). However, in some studies (Ashraf et al., 2009, Dong , 2010), BP levels remained unchanged with the improvement in serum 25-hydroxyvitamin D (25(OH)D) levels. Given the equivocal findings relating vitamin D status with lipid profiles and BP in children, our aim was to study the influence of vitamin D status on BMI, serum lipids and blood pressure in a longitudinal cohort of adolescents living in the Johannesburg Metropolitan area (latitude 26°S, altitude 1600 m), South Africa. We hypothesised that an increase in vitamin D status would be associated with a reduction in risk of non-communicable diseases.

6.4 Methods

6.4.1 Study subjects

Children from the Bone Health subcohort of the Birth to Twenty longitudinal study in Johannesburg were studied. The Birth to Twenty cohort consists of subjects who have been followed since their birth in 1990. A total of 3273 black and white singleton deliveries were enrolled within the Greater Johannesburg Metropolitan area over a seven-week period. Children in the Bone Health cohort have since the age of 9 years been investigated annually for factors affecting bone growth and bone mass accrual. Children with chronic illnesses, or on medication or drugs and mineral supplements known to affect bone metabolism were excluded from participating in the study.

Black and white children were measured for anthropometry (weight, height and BMI), BP (systolic blood pressure (SBP) and diastolic blood pressure (DBP)) and 25(OH)D levels at ages 11, 12 13, 15 and 18-20 years. Lipid levels, total cholesterol (TC), triglycerides (Trig), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) were only measured in years 12 and 18-20. For the longitudinal assessment of age related changes in serum lipids, only participants, who had measurements at both years 12 and 18-20, were included, resulting in the analysis of 200 participants. The data for longitudinal analysis of anthropometry, BP and 25(OH)D using the generalised estimating equation (GEE) comprised of the following groups of subjects: Year 11 (N = 288), Year 12 (N = 253), Year 13 (N = 292), Year 15 (N = 238) and Year 18-20 (N = 368). Eight subjects, who only had measurements taken once during the study, were excluded from the GEE data analysis. For participants aged 11 to 15 years, the BMI for age cut-offs for underweight or healthy weight were defined as $< 85^{th}$ percentile and $\geq 85^{th}$ percentile for overweight or obese subjects (Barlow et al., 2007). For participants aged 18-20 years, underweight or normal weight was defined as a BMI of < 25 kg/m^2 and overweight or obese as a BMI of ≥ 25 kg/m^2 (Cole et al., 2000). Age and height adjusted reference values were used to define normal BP in children aged 11 - 15 years (Flynn et al., 2017), while in participants aged 18-20 years normal BP was defined as SBP ≤ 140 mm Hg and DBP ≤ 90 mmHg. Cut-offs for normal lipid profiles were TC ≤ 5 mmol/L, Trig ≤ 1.7 mmol/L, LDL-C ≤ 3.0 mmol/L, HDL-C ≥ 1.0 mmol/L in males and ≥ 1.2 mmol/L in females (Juhola et al., 2011). The BP and lipid profile were further categorised as binary variables as

follows; BP status (0 = abnormal BP and 1 = normal BP) and dyslipidaemia status (0 = abnormal lipid profile and 1 = normal lipid profile)

The study protocol was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand, Johannesburg (Approval no. M980810). Consent was obtained from the adolescents' guardians and assent from the participants.

6.4.2 Anthropometric and blood pressure measurements

Body height in centimetres (cm) and weight in kilograms (kg) were measured by trained research assistants. Height was measured in bare feet to the nearest 0.1 cm using a rigid stadiometer and weight was measured in subjects wearing light clothes without shoes to the nearest 0.1 kg using a calibrated scale balance. BMI was calculated by dividing weight in kilograms by height in metres squared (kg/m^2).

SBP and DBP (mmHg) were measured at the right upper arm using an automated oscillometric device (Omron M6 HEM-7001-E, Omron Corporation, Kyoto, Japan). Prior to BP readings, each subject rested quietly for 5 minutes. After each reading the cuff was automatically deflated to 0 mmHg. BP was measured three times with the subject seated. The average of the three readings was used for systolic and diastolic blood pressure.

6.4.3 Biochemical measurements and calculations

Blood samples (20 ml) were obtained after an overnight fast from each subject. The blood samples for 25(OH)D and lipid concentrations were collected into plain red top tubes. The blood was allowed to clot at room temperature for a minimum of 20 minutes, before being centrifuged, and the serum separated and stored at -80^0C. Serum 25(OH)D was measured by a chemiluminescence assay using the Diasorin Liaison instrument (Diasorin Liaison, Stillwater, MN, USA) (intra-assay and inter-assay variations were < 5% and < 8% respectively). Our laboratory participates in an international quality assurance programme (DEQAS - International Vitamin D External Quality Assessment Scheme, London, United Kingdom). Since our participation in DEQAS external quality control, our laboratory had received the certificates of efficiency on a yearly basis (i.e 80% or more results fell within 30% of the all laboratory trimmed mean). Lipids were measured by a Randox Daytona Instrument (Randox Laboratories Ltd, County Antrim, UK) (intra and inter-assay variation < 5%).

6.4.4 Statistical analyses

The data was analyzed using STATA software package version 11 (StataCorp, College Station, Texas, US). All continuous variables were presented as means and standard deviations (mean ± SD). Because of missing data at each longitudinal time point, the association of 25(OH)D levels with risk factors was determined using GEE which accommodates missing values and allows repeated measures. The bivariate GEE models assessed the relationship of 25(OH)D with BMI, BP and lipid profiles, the covariates (age, gender, ethnicity and season) were controlled for in multivariate GEE models.

6.5 Results

The anthropometric and biochemical data are shown in Tables 6.1 and 6.2. There was a significant increase in BMI and BP and a significant decrease in 25(OH)D with age during the 10 years of study (year 11, 12, 13, 15 and 18-20). Serum 25(OH)D, TC and LDL-C were higher in males at 12 years of age than at 18-20 years of age. In female participants only 25(OH)D was higher when they were younger. Over the period from 12 years of age to 18-20 years, 25(OH)D was found to be significantly negatively associated with LDL-C, when adjusted for age, BMI, gender, ethnicity and season of the year.

Table 6.1: Cross-sectional assessment of the BMI, blood pressure and vitamin D status at different time points in a study population.

Variables	BMI **(kg/m^2)** **Total**	***Abnormal BMI (kg/m^2)**	SBP **(mmHg)** **Total**	**#Abnormal SBP (mmHg)**	DBP **(mmHg)** **Total**	**#Abnormal DBP (mmHg)**	25(OH)D **(nmol/L)** **Total**	Vitamin D deficiency (25(OH)D < 50 nmol/L)
Year 11	18 ± 2.9	Female (n = 117)	100 ± 10.1	Female (n = 133)	65 ± 8.2	Female (n =133)	73 ± 30.8	Female (n = 135)
	N=256	**31 (26%)**	**N = 283**	**3 (2%)**	**N = 283**	**13 (10%)**	**N = 288**	**41 (30%)**
		Male (n = 139)		Male (n =150)		Male (n = 150)		Male (n= 153)
		12 (9%)		**1 (1%)**		**6 (4%)**		**34 (22%)**
		Mean (%) = 17%		**Mean (%) = 1.4%**		**Mean (%) = 7%**		**Mean (%) = 26%**
Year 12	19 ± 3.1	Female (n = 119)	105 ± 10.5	Female (n = 95)	68 ± 7.9	Female (n = 95)	81 ± 25.7	Female (n = 124)
	N=253	**29 (24%)**	**N = 211**	**2 (2%)**	**N=211**	**22 (23%)**	**N=261**	**16 (13%)**
		Male (n = 134)		Male (n = 116)		Male (n = 116)		Male (n = 137)
		16 (12%)		**2 (2%)**		**7 (6%)**		**7 (5%)**
		Mean (%) = 18%		**Mean (%) = 2%**		**Mean (%) = 14%**		**Mean (%) = 9%**
Year 13	20 ± 3.7	Female (n = 138)	108 ± 10.5	Female (n =102)	71 ± 8.6	Female (n = 102)	70 ± 23.3	Female (n = 139)
	N=291	**36 (26%)**	**N=226**	**6 (6%)**	**N=226**	**32 (31%)**	**N = 292**	**26 (19%)**
		Male (n= 153)		Male (n =124)		Male (n = 124)		Male (n = 153)
		22 (14%)		**3 (2%)**		**13 (10%)**		**22 (14%)**
		Mean (%) = 20%		**Mean (%) = 4%**		**Mean (%) = 20%**		**Mean (%) = 16%**
Year 15	21 ± 3.7	Female (n = 117)	109 ± 11.8	Female (n = 104)	69 ± 7.5	Female (n = 104)	62 ± 18.5	Female (n = 139)
	N=236	**29 (25%)**	**N = 214**	**3 (3%)**	**N = 214**	**4 (4%)**	**N=238**	**26 (19%)**
		Male (n = 119)		Male (n = 110)		Male (n = 110)		Male (n = 153)
		13 (11%)		**1(1%)**		**2 (2%)**		**22 (14%)**
		Mean (%) = 18%		**Mean (%) = 2%**		**Mean (%) = 3%**		**Mean (%) = 20%**

Year 18-20	23 ± 4.3 **N = 339**	Female (n = 159) **57 (36%)** Male (n = 180) **28 (16%)** **Mean (%) = 25%**	119 ± 11.6 **N = 327**	Female (n = 155) **3 (2%)** Male (n = 172) **8 (5%)** **Mean (%) = 3%**	80 ± 17.5 **N = 327**	Female (n = 155) **32 (21%)** Male (n = 172) **42 (24%)** **Mean (%) = 23%**	53 ± 17.6 **N = 368**	Female (n = 173) **94 (54%)** Male (n = 194) **62 (32%)** **Mean (%) = 42%**
p-value and overall prevalence	P-value (Anova) **0.0001**	Prevalence = 273 (**20%)**	P-value (Anova) **0.0001**	Prevalence = 32 (**3%)**	P-value (Anova) **0.0001**	Prevalence = 173 (**14%)**	P-value (Anova) **0.0001**	Prevalence = 350 (**23%)**

*Abnormal BMI for Year 11 to Year 15, $\geq 85^{th}$ percentiles for age and sex, and abnormal BMI for Year 18 – 20, ≥ 25 kg/m^2.

#Hypertension status for Year 11 to Year 15, $\geq 95^{th}$ percentile for age, sex, and height and for Year 18 – 20, SBP $\geq$ 140 mm Hg and DBP $\geq$ 90 mmHg

**25(OH)D < 50 nmol/L = vitamin D deficiency, $\geq$ 50 nmol/L = vitamin D sufficiency

Over a period of 10 years, there was a significant relative increase of mean BMI, SBP and DBP (all p-values < 0.0001) and a significant decrease in 25(OH)D (p-value < 0.0001) with age in study participants. The overall prevalence of abnormal BMI and vitamin D deficiency was 273 (20%) and 350 (23%) respectively. There was also an overall low prevalence of abnormal SBP (n = 32 (3%)) as compared to abnormal DBP (n = 173 (14%).

Change in blood pressure, anthropometry, body composition and biochemical variables in adolescents between the ages of 12 and 18 - 20 years stratified by sex is shown in table 6.2.

Table 6.2: Change in blood pressure, anthropometry, body composition and biochemical variables in adolescents between the ages of 12 and 18 - 20 years stratified by sex.

	Female					Male				
Variables	Year 12 (N = 88)	Abnormal (%)	Years 18-20 (N = 88)	Abnormal (%)	p-value*	Year 12 (N = 112)	Abnormal (%)	Years 18-20 (N = 112)	Abnormal (%)	p-value
Systolic BP	106 ± 9.5	1 (1%)	112 ± 8.4	0 (0%)	**0.0001**	105 ± 10.3	2 (2%)	121 ± 10.6	6 (5%)	**0.0001**
Diastolic BP	70 ± 8.0	15 (17%)	70 ± 7.9	9 (10%)	0.64	67 ± 7.5	6 (5%)	72 ± 7.9	31 (28%)	**0.003**
Height (cm)	153 ± 8.5		161 ± 6.8		**0.0001**	149 ± 8.3		172 ± 7.9		**0.0001**
Weight (kg)	46 ± 10.8		63 ± 14.3		**0.0001**	41 ± 8.0		63 ± 10.2		**0.0001**
BMI (kg/m^2)	19 ± 3.6	21 (24%)	24 ± 5.0	36 (41%)	**0.0001**	18.0 ± 2.5	14 (13%)	21 ± 2.8	11 (10%)	**0.0001**
25(OH)D (nmol/L)	72 ± 22.8	13 (15%)**	47 ± 19.2	51 (58%)**	**0.0001**	84 ± 26.4	6 (5%)**	58 ± 15.6	7 (5%)**	**0.0001**
Total Cholesterol (mmol/L)	4.21 ±0.95	14 (16%)	4.14 ± 1.1	10 (11%)	0.63	4.28 ± 0.8	19 (17%)	3.86 ± 0.7	7 (6%)	**0.0003**
Triglycerides (mmol/L)	0.83 ± 0.4	6 (7%)	0.74 ± 0.4	3 (3%)	0.08	0.77 ± 0.3	8 (2%)	0.76 ± 0.3	1 (1%)	0.78
HDL-C (mmol/L)	1.22 ± 0.3	15 (17%)	1.3 ± 0.3	20 (23%)	0.34	1.20 ± 0.3	25 (22%)	1.14 ± 0.3	35 (31%)	0.14
LDL-C (mmol/L)	2.30 ± 0.80	12 (14%)	2.11 ± 0.82	12 (14%)	0.11	2.40 ± 0.7	21 (19%)	2.01 ± 0.6	9 (8%)	**0.0001**

*p values were calculated using the paired Student t-test

**25(OH)D < 50 nmol/L = vitamin D deficiency, ≥ 50 nmol/L = vitamin D sufficiency

Table 2: shows comparisons in the same subjects at age 12 years and at age 18-20 years. In female subjects, SBP was significantly higher in participants aged 18-20 years, while 25(OH)D levels were significantly lower. There was no significant change in lipid levels in the females at the two ages. In males the pattern was similar to that of females over time with increases in BP. 25(OH)D levels were significantly lower in the group of males aged 18-20 years than at 12 years of age. TC and LDL-C were significantly lower in males at 18-20 years of age compared to when the subjects were 12 years old, with the triglycerides remaining constant over the period. There was a slight decrease in abnormal DBP in female participants from 15 (17%)(younger) to 9 (10%)(older), which is in contrast to the males, in whom abnormal DBP increased from 6 (5%)(younger) to 31 (28%)(older). Younger participants had similar percentages of abnormal TC (females 14 (16%) and males 19 (17%), furthermore younger males had abnormally increased LDL-C in 21 (19%) compared to that in older males 9 (8%).The prevalence of abnormal HDL-C in female ranged from 15(17%) (younger) to 20 (23%) (older) and in males from 25(22%) (younger) to 35(31%)(older). There was 43% increase in vitamin D deficiency in females as compared to 1% increase of vitamin D deficiency on males.

The GEE bivariate and multivariate regression analysis of 25(OH)D with blood pressure, BMI (over a 10 year period) and lipids (at 12 and 18-20 years) is shown in table 6.3.

Table 6.3: The GEE bivariate and multivariate regression analysis of 25(OH)D with blood pressure, BMI (over a 10 year period) and lipids (at 12 and 18-20 years).

Cardio-metabolic risk Factors Versus 25(OH)D	BMI β(p-value)	SBP (mmHg) β(p-value)	DBP (mmHg) β(p-value)	BP status 0=Abnormal BP 1=Normal BP	TC (mmol/L) β(p-value)	Trig (mmol/L) β(p-value)	HDL-C (mmol/L) β(p-value)	LDL-C (mmol/L) β(p-value)	Lipid status 0= Abnormal Lipid Profile 1 = Normal BP
Bivariate Analysis	-1.59 (**0.0001**)	-0.32 **(0.0001)**	-0.31 **(0.0001)**	6.85 **(0.001)**	1.11 (0.26)	5.13 **(0.04)**	-5.11 (0.06)	-1.18 (0.34)	2.51 (0.25)
Model 1[a] (multivariate-Unadjusted)	____	-0.08 (0.56)	-0.17 **(0.01)**	-3.35 (0.35)	1.84 (0.11)	5.02 (0.09)	-4.51 **(**0.15**)**	-2.49 (0.08)	3.61 (0.15)
Model 2[b] (multivariate-Adjusted)	____	-0.03 (0.85)	0.09 (0.92)	-1.11 (0.75)	0.24 (0.82)	0.05 (0.98)	-5.8 (0.05)	-3.7 (**0.01**)	3.04 (0.20)

Model 1[a] – multivariate analysis

Model 2[b] – multivariate analysis adjusted for age, BMI, gender, ethnicity and season of the year

In GEE bivariate regression analysis, 25(OH)D was significantly negatively associated with BMI, SBP, DBP and positively associated with triglyceride levels and better BP status. In the unadjusted multivariate model, 25(OH)D was only inversely associated with DBP. After adjustment for possible confounding variables (age, BMI, gender, ethnicity, and season) in Model 2[b], 25(OH)D was only significantly inversely associated with LDL-C.

6.6 Discussion

6.6.1 Summary of the present study results

Unlike other studies reporting higher percentages of hypovitaminosis D (Lamberg-Allardt, 2012), the present Bone Health cohort had vitamin D deficiency (25(OH)D < 50 nmol/L) in 17% of the participants (age 11 – 15 years), and 42% of participants (age 18 – 20 years) (Holick, 2007, Heaney, 2013). Interestingly, the prevalence of vitamin D deficiency was 26% in year 11, which was higher than when the participants were 10 years old (7% deficiency prevalence). The reason for the differences in 25(OH)D deficiency between year 10 and 11 participants is not known as both group samples were run under the same laboratory conditions and in duplicates. There was significant increase in repeated measures of mean BMI, and BP, while there was a decrease in 25(OH)D over a period of 6-8 years, which were the expected findings in children going through adolescence (Reis et al., 2009). Lipid levels measured in 12 and 18-20 year old participants were significantly higher in the male cohort at 12 years of age than at 18-20 years of age. These differences were not seen in female cohort. Over a period of 6-8 years, 25(OH)D was found to be significantly negatively associated with LDL-C (Model 2^{b}), which may suggest that increased 25(OH)D levels are associated with favourable lipid profiles (Kelishadi et al., 2014, Williams et al., 2014).

6.6.2 Association of 25(OH)D with body composition

25(OH)D is by nature hydrophobic, hence is reported to be easily sequestrated into the body fat cells which may result in vitamin D deficiency (Wortsman et al., 2000). Despite vitamin D deficiency being traditionally linked to altered bone mineral metabolism (Holick, 2006), low 25(OH)D levels have been found to be negatively associated with BMI and body composition (total and percentage fat mass, visceral adipose tissue) (Gilbert-Diamond et al., 2010, Dong et al., 2010a, Rajakumar et al., 2011a). In the present study, the females were found to have twice abnormal BMI as compared to their male peers, which may suggest that black females are at risk of vitamin D deficiency. 25(OH)D was found to be inversely associated with BMI, before (β = -1.59, p-value = 0.0001) and after (β = -0.74, p-value =0.0001) adjustment for other variables (age, gender, ethnicity and season). This may reflect an independent association of BMI with 25(OH)D and the sequestration of 25(OH)D in the body fat adipocytes as reported by other studies (Wortsman et al., 2000, Arunabh et al., 2003b). A study by Blum et al., 2008 has shown a strong inverse relationship between amount of fat tissue and serum vitamin D_3, but the researchers failed to show correlation between body

weight and vitamin D_3 because of small sample size (N=17). However, other researchers have found total body volume to have an important mechanism in the explanation of the relationship (Drincic et al., 2012). This is because according to Drincic et al., 2012 dilution of ingested or cutaneously synthesized vitamin D in the large fat mass of obese patients may fully explain their typically low vitamin D status. Another possible explanation for low vitamin D in obesity has been described in animal studies, in which obesity was associated with a down regulation of CYP2R1, the major hepatic hydroxylase responsible for the conversion of vitamin D to 25(OH)D (Bouillon and Bikle, 2019).

6.6.3 Association of 25(OH)D with blood pressure

Vitamin D deficiency has been reported to be associated with diseases that are not bone related and hypertension is one of them (Forman et al., 2007, Kao et al., 2015). The importance of vitamin D in hypertension has also been investigated in animal studies where vitamin D was shown to reduce blood pressure through inhibition of the renin-angiotension system (Li, 2003). Researchers have found low 25(OH)D to be associated with increased blood pressure in longitudinal and cross-sectional studies around the globe (Forman et al., 2007, Reis et al., 2009, Kao et al., 2015). But no association was found in randomised clinical trials and case-control studies (Dong et al., 2010b, Olson et al., 2012). In the present study, an inverse relationship between 25(OH)D and BP was found in bivariate linear regression analysis but the relationship disappeared after adjustment of age, BMI, gender, ethnicity, and season. Hence, researchers have suggested that understanding the relationship between 25(OH)D and cardiometabolic risk factors is a challenge because of multiple variables that may confound the association (Dolinsky et al., 2013). More insight into this relationship could be provided by randomised clinical trials which directly interrogate the relationship of 25(OH)D with cardiometabolic risk factors.

6.6.4 Association of 25(OH)D with lipids

Cross-sectional and a few prospective studies have reported that the association of 25(OH)D and abnormal lipid levels was related to obesity in children and adults, which may lead to cardiovascular complications (Wang et al., 2008, Stokić et al., 2015, Kumaratne et al., 2017). The TC and LDL-C in younger male children were found to be higher as compared to their older male peers, of which the reason is not known. Hence, it will need to be further investigations in studies in similar settings to us using bigger samples sizes. The present study found 25(OH)D to be significantly positively related to triglyceride levels in bivariate

analysis and negatively associated with LDL-C in multivariate analysis (Model 2^b). This may be the result of sufficient vitamin D status in the present study population, which may be associated with favourable lipid profile. However, these findings have not been consistently found in children and adolescents (Nam et al., 2014, Kumaratne et al., 2017).

There are several limitations associated with the study; loss to follow up over the 6-8 years of study resulted in a reduction in subjects with measurements at both 12 and 16-18 years and no information was collected on time spent outdoors or of physical activity and sunscreen use. The study used BMI as a measure of fat mass, which is unable to distinguish lean mass from fat mass. The present study was restricted to the Greater Johannesburg metropolitan area; hence the findings cannot reflect the general population in South Africa.

Conclusion: Although we were able to show relationships using bivariate analyses between vitamin D status and BMI, BP, and lipid levels over a 6-10 year period during adolescence in a cohort of black and white South Africans, the significant relationships mostly disappeared following adjustment for covariates, except for the relationship between 25(OH)D and BMI, which persisted after adjustment. One possible reason for lack of relationships with 25(OH)D might relate to the adequate vitamin D status of most of the subjects.

Acknowledgements: The authors acknowledge the South African Medical Research Council (SAMRC), Welcome Council, Trust (United Kingdom) and Vice Chancellor's award, University of the Witwatersrand, Johannesburg for funding the research. The contribution of Bone Health and Birth to Twenty staff members presently Developmental Pathways for Health Research Unit (DPHRU) in data collection both past and present is greatly appreciated by the authors. Special thanks to study participants and theirs parents for participating in our data collection.

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CHAPTER 7: Discussion Summary

7.1 Preface to the chapter

The chapter's main emphasis is on key findings of the thesis, with focus on public health implications, strengths and limitations.

7.2 Summary of findings

The main aims of the thesis were to i) assess vitamin D status in 10-year-old children living in the greater Johannesburg metropolitan area, ii) determine longitudinally if there is vitamin D tracking in adolescent children over a period of 10 years, and iii) evaluate the association of vitamin D status with non-communicable disease risk in children over 10 years. The consolidated findings in line with the empirical chapters are outlined on the table 7.1 (page 130).

Table 7.1 Consolidated study findings

Chapter	Objectives	Key findings
4	To determine vitamin D status in 10-year-old children and factors influencing it.	For the overall study population, 7% had vitamin D deficiency (< 50 nmol/L), 19% vitamin D insufficiency (50 – 74 nmol/L) and 74% was vitamin D sufficient (≥ 75 nmol/L). Hence, vitamin D supplementation or fortification should not be considered in healthy South African children living in Johannesburg. 25(OH)D levels were significantly higher in white than black children during autumn and summer months. Seasonal variations in 25(OH)D were found in white children, with values being highest in summer and autumn. No seasonal variations were noted in black children. The season ($\beta = 0.28$, $p = 0.0001$), ethnicity ($\beta = -0.29$, $p = 0.0001$), sex ($\beta = -0.16$, $p = 0.003$), and total fat mass ($\beta = -0.15$, $p = 0.01$) were the only factors found to have an influence on 25(OH)D.
5	To determine whether tracking of vitamin D status occurs in healthy adolescents, we assessed the variability of 25-hydroxyvitamin D (25(OH)D) every 2 years over a 10 year period in a longitudinal	No significant correlation between 25(OH)D in the earlier and later years of adolescence was found, although there were significant correlations between year 11 and year 13 ($r = 0.71$; $p < 0.0001$), and between years 15, 17 and 20 ($r \geq 0.65$; $p < 0.0001$). The study suggests that a single measurement of 25(OH)D may not accurately reflect the vitamin D status of an individual over a

	cohort of adolescents living in Johannesburg, South Africa (latitude 26°S).	prolonged period of time. These results cast doubt on the veracity of reports that suggest that vitamin D status (as measured on a single occasion) may influence long term disease outcomes.
6	To determine whether vitamin D status is associated with non-communicable disease risk in children.	There was evidence of changes in 25(OH)D, BMI and BP in adolescents over a period of 10 years. After controlling for covariates, increased 25(OH)D was significantly associated negatively with LDL-C, which may be related to favourable lipid profile in children and adolescents.

7.3 Hypothesis revisited

7.3.1 Hypothesis 1

Children living in Johannesburg would generally be vitamin D sufficient as Johannesburg has greater than 8 sunshine hours daily throughout the year, however black children in general would have lower 25(OH)D concentrations than white children due to their greater skin pigmentation.

We were able to reject the null hypothesis as black children were found to have significantly lower 25(OH)D levels than their white peers. Furthermore, the majority of children (74%) had sufficient 25(OH)D levels.

7.3.2 Hypothesis 2

Vitamin D status in children living in Johannesburg will track during adolescence because of abundance of sunlight.

We were able to reject the null hypothesis as vitamin D status changed significantly in just over half the children between early and late adolescence.

7.3.3 Hypothesis 3

An increase in vitamin D status would be associated with a reduction in risk of non-communicable diseases.

We were able to reject the null hypothesis as 25(OH)D levels were significantly negatively associated with LDL-C after adjusting for age, ethnicity, sex and adiposity. Thus an adequate vitamin D status may be related to a more favourable lipid profile in children and adolescents.

7.4 Public health implications

One finding that was eminent in the present study was a high percentage of subjects with vitamin D sufficiency throughout the year as defined by either IOM or The Endocrine Society (Ross et al., 2011a, Holick et al., 2011, Rosen et al., 2012). Hence, it is suggested that food supplementation or fortification is unnecessary in healthy South African children living in Johannesburg outside of the inner-city area. It is however not known if the results can be transferred to other more southerly regions in South Africa due to differences in latitude and cloud cover between Johannesburg and these regions. Furthermore, no information was obtained on possible at-risk groups, such as infants in general and particularly those who are breastfed for prolonged periods, or in children living in the high-rise inner-city areas, where overcrowding and lack of open spaces for children to play might increase the risk of vitamin D deficiency.

Another finding of importance relates to the finding of the lack of vitamin D status tracking over the period of adolescence. At present, there are only a handful of studies that have assessed vitamin D status over a prolonged period (Jorde et al., 2010c, Berger et al., 2012, Faridi et al., 2017). The study on vitamin D tracking was undertaken in adolescent children, which are in a critical stage of changes in lifestyle, possibly leading to reduced cutaneous synthesis of vitamin D because of spending less time outdoors.

Vitamin D tracking was present in early (year 11 and 13 participants) and late adolescence (year 15, 17 and 20 participants) but not throughout adolescence. The reasons for the difference in tracking are not known but it is unlikely to be due to laboratory variations as all samples for a single participant were measured in a single assay run. Thus to better understand the relationship between vitamin D status and various disease conditions, longitudinal studies are required in which 25(OH)D levels are measured throughout the study

period to report on the overall pattern of vitamin D tracking in subjects in relation to the development of the disease being studied.

A third finding, that of the chronic disease risk factors studied in the adolescents, 25(OH)D levels were only associated with LDL-C in children over 10 years. There was evidence of changes in BMI, blood pressure and lipid profile over a period of time, but these were unassociated with vitamin D status after adjusting for possible confounding variables. Hence, we are suggesting that increased 25(OH)D may be associated with better lipid profiles in this age group of children, but the study was hampered by the generally good vitamin D status of most children. More studies with larger sample size to assess the longitudinal association of 25(OH)D with NCD risk are warranted.

7.5 Strengths and limitations

7.5. 1 Vitamin D measurement in the laboratory

Due to challenges in the laboratory measurement of 25(OH)D (Snellman et al., 2010), the present study used chemiluminescence assay which had a turnaround time of 35 minutes per sample with better intra and inter coefficient of variation (< 10%) than a number of other types of assays. 25(OH)D was measured by an assay manufactured by Diasorin which introduced a chemiluminescence immunoassay on their "Liasorin" automated immunoassay platform in 2004. The assay was updated to have improved sensitivity and precision and was renamed Liasion Total in 2007 (Wallace et al., 2010). Despite HPLC and LC-MS/MS methods still being the preferred techniques for quantitatively measuring vitamin D status (Schleicher et al., 2011, Farrell et al., 2012), the Diasorin assay is used extensively worldwide, thus allowing comparison of the results from these studies with others throughout the world. In general, precision of immunoassay, HPLC and LC-MS/MS are comparable and all have the required sensitivity to identify severe vitamin D deficiency (Wallace et al., 2010, Gallelli et al., 2019).

For validation of results, our laboratory participates in an international quality assurance programme (DEQAS - International Vitamin D External Quality Assessment Scheme, London, United Kingdom). Since our participation in DEQAS external quality control, our laboratory had received the certificates of efficiency on a yearly basis (i.e 80% or more results fell within 30% of the all laboratory trimmed mean).

7.5.2 Study designs

Studies vary in terms of their strengths and limitations due to their study designs. This section of the thesis will examine the strengths and limitations related to each study in table 7.2 below.

Table 7.2 Strengths and limitations

Factors influencing vitamin D status in 10-year-old children in Johannesburg, South Africa.	
Strengths	**Limitations**
The 25(OH)D was measured in duplicates and Diasorin Liason machine was calibrated daily for running every batch of samples. All the intra and inter-assay co-efficient of variation was less than 10%.	The 25(OH)D levels and the factors influencing the levels were assessed cross-sectionally thus no cause and effect could be deduced from the associations found. The number of white children in the study was relatively small reducing the strength of the associations.
Does vitamin D track in adolescent children?	
Strengths	**Limitations**
The 25(OH)D levels were measured in duplicates in samples collected over 10 years. All samples from one individual were measured in the same assay, thus negating inter-assay variability.	Of the 504 children, only 99 children met the criteria for inclusion in the present study. They were required to have blood samples taken on 3 or more occasions during the longitudinal study. The majority of children excluded had blood sampled less frequently due to various factors.
Is vitamin D status associated with non-communicable disease risk in children?	
Strengths	**Limitations**
Repeat measures of 25(OH)D over a 10 year period were associated with NCD risk in children using the Generalised Estimating Equation (GEE) which has the advantage of accommodating missing values.	Loss to follow up over the 6-8 years of study resulted in a reduction in subjects with measurements at both 12 and 16-18 years and no information was collected on time spent outdoors or of physical activity and sunscreen use. The study used BMI as a measure of fat

	mass, but its shortcoming is that it cannot distinguish lean mass from fat mass. The present study was restricted to the Greater Johannesburg metropolitan area; hence the findings may not reflect the general population in South Africa.

7.6 Novel study findings

Understanding of vitamin D physiology has for a long time been confined to bone health (Holick, 1981, Holick, 1989). One of the important finding of the present study was the presence of vitamin D sufficiency (25(OH)D > 50 nmol/L) in the majority of participants. The current study also added to the literature on vitamin D research by associating 25(OH)D with NCD risk factors at longitudinal level; it was suggested that vitamin D status of our study participants might be associated with more favourable lipid profiles in children and adolescents.

Researchers have measured vitamin D status only once in most studies in which the association of vitamin D status with disease risk have been examined. In the present study we tracked vitamin D status over a period of time. Only a handful of studies in this field have been published with contrasting results. The present study found no significant correlation between 25(OH)D in earlier and later years of adolescence. Hence it was suggested that measurement of 25(OH)D at a single time point does not reflect the long term status of vitamin D in adolescents.

7.7 Future research

We do not know if our present study findings may hold true in other parts of South Africa and other countries in southern Africa. However, relatively good vitamin D status (25(OH)D > 50 nmol/L) in the vast majority of adolescents is encouraging, but further studies across the life-course are required and particularly in possible at-risk groups (infants, inner-city dwellers and the elderly).

Furthermore, longitudinal randomised controlled trials (RCTs) and systematic reviews and meta-analysis of RCTs studies with larger sample sizes are warranted to further unpack

vitamin D tracking and its association with NCD risk. Changes in 25(OH)D levels are reported as children grow older, hence future studies must take into consideration in their statistical modelling the behavioural changes that may occur as time progresses.

In conclusion, the present study population had sufficient vitamin D status, 25(OH)D > 50 nmol/L. Higher 25(OH)D in the study population was associated with a favourable lipid profile. The lack of long-term tracking during adolescence is an important finding and casts doubt on epidemiological studies of NCDs in which vitamin D status is only measured once and used as a measure of vitamin D status over a period of time.

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Appendix I - Total Cholesterol

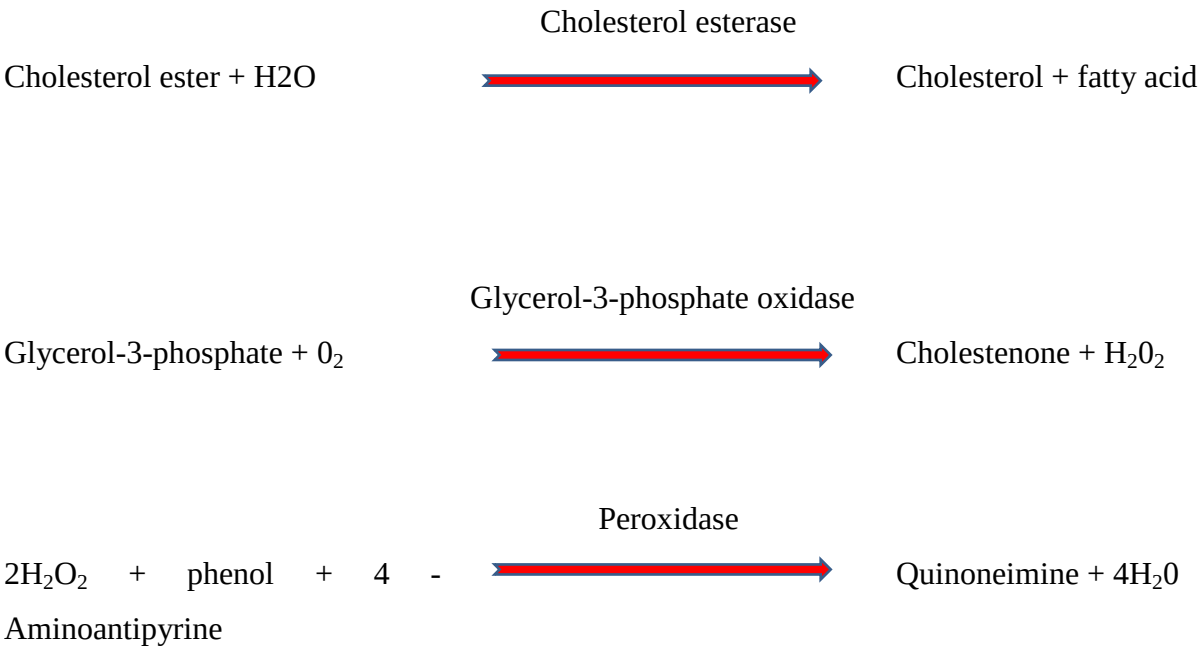
Total Cholesterol (Instrument = RX Daytona)

Clinical relevance

Cholesterol measurements are used in the diagnosis and treatment of lipid lipoprotein metabolism disorders. Lipids play an important role in the body by serving as hormones or hormone precursors, aid in digestion, provide energy, storage and metabolic fuels, act as functional and structural components in bio-membranes and form insulation to allow nerve conduction and prevent heat loss.

Assay Principle

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.



Appendix II - Triglycerides

Triglycerides (Instrument = RX Daytona)

Clinical relevance

Triglyceride measurements are used in the diagnosis and treatment of diseases involving lipid metabolism and various endocrine disorders such as diabetes mellitus, nephrosis and liver obstruction.

Principle

Triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipases}} \text{Cholesterol} + \text{fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Cholestenone} + H_2O_2$$

$$\text{Glycerol-3-phosphate} + 0_2 \xrightarrow{\text{Glycerol-3-phosphate oxidase}} \text{Quinoneimine} + 4H_20$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + \text{HCL} + 4H_20$$

Appendix III - Direct Low Density Lipoprotein Cholesterol

Direct Low Density Lipoprotein Cholesterol (LDL-C) (Instrument = RX Daytona)

Clinical relevance

Low density lipoproteins (LDL) are synthesized in the liver by the action of various lipolytic enzymes on triglyceride rich Very Low Density Lipoproteins (VLDLs). Specific LDL receptors exist to facilitate the elimination of LDL from plasma by liver parenchymal cells. It has been shown that most of the cholesterol stored in atherosclerotic plaques originates from LDL. For this reason the LDL-Cholesterol concentration is considered to be the most important clinical predictor of all single parameters, with respect to coronary atherosclerosis. Accurate measurement of LDL-Cholesterol is of vital importance in therapies which focus on lipid reduction to prevent atherosclerosis or reduce its progress and to avoid plaque rupture.

Principle

Reagent composition (**Enzyme Reagent R1** + **Enzyme Reagent R2**)

The assay consists of two reaction steps:

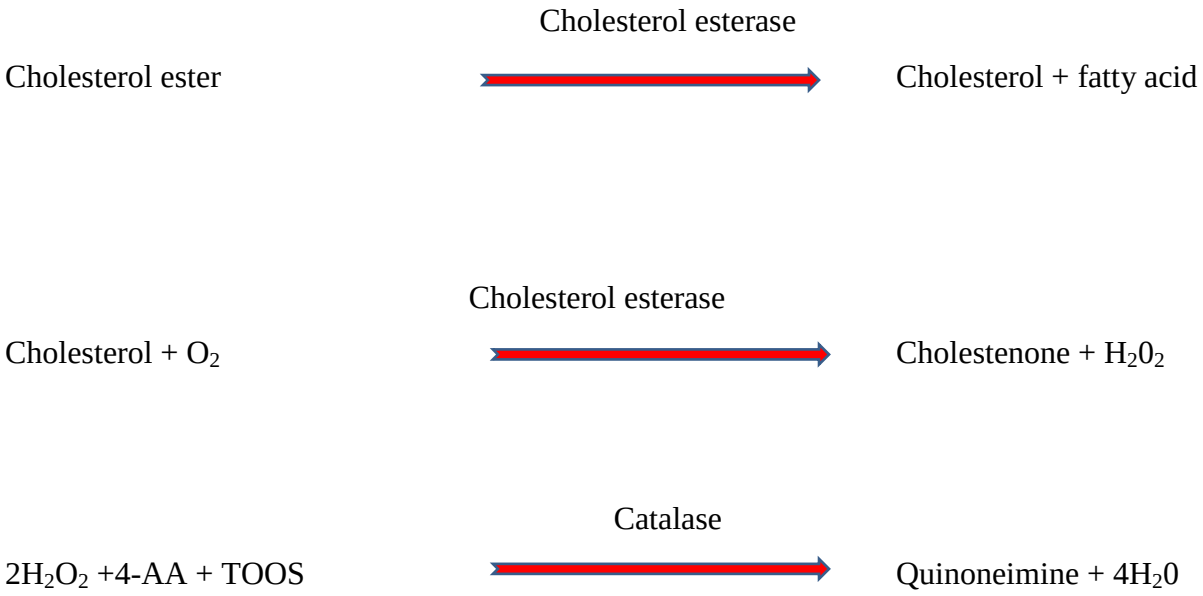
1. Elimination of chylomicron, VLDL-Cholesterol and HDL-Cholesterol by cholesterol esterase, cholesterol oxidase and subsequently catalase

Cholesterol ester $\xrightarrow{\text{Cholesterol esterase}}$ Cholesterol + fatty acid

Cholesterol + O_2 $\xrightarrow{\text{Cholesterol esterase}}$ Cholestenone + H_20_2

$2H_2O_2$ $\xrightarrow{\text{Catalase}}$ $2H_20 + 0_2$

2. Specific measurement of LDL-Cholesterol after release of LDL-Cholesterol by detergents in Reagent 2.



The intensity of the quinoneimine dye produced is directly proportional to the cholesterol concentration when measured at 600nm

4-AA = Aminoantipyrine

TOOS = N-Ethy-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline

Appendix IV - Direct HDL-Cholesterol

Direct HDL-Cholesterol (Instrument = RX Daytona)

Clinical relevance

High density lipoproteins (HDL-C) are one of the major classes of plasma lipoproteins. They are composed of a number of heterogeneous particles, including cholesterol and vary with respect to size and content of lipid and apolipoprotein. HDL serves to remove cholesterol from the peripheral cells to the liver, where the cholesterol is converted to bile acids and excreted into the intestine. An inverse relationship between HDL-Cholesterol (HDL-C) levels serum and the incidence/prevalence of coronary heart disease (CHD) has been demonstrated in a number of epidemiological studies.

Accurate measurement of HDL-C is of vital importance when assessing patient risk from CHD. In this diagnostic test kit a method for direct measurement of HDL-C, without sample pretreatment, is presented. Direct measurement gives improved accuracy and reproducibility when compared to precipitation methods.

Principle

Reagent composition (**Enzyme Reagent R1** + **Enzyme Reagent R2**)

The assay consists of two reaction steps:

1. Elimination of chylomicron, VLDL-Cholesterol and HDL-Cholesterol by cholesterol esterase, cholesterol oxidase and subsequently catalase

$$\text{Cholesterol ester} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol + fatty acid}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\text{Cholesterol esterase}} \text{Cholestenone + } H_20_2$$

Catalase

$2H_2O_2$ ⟶ $2H_20_2 + O_2$

2. Specific measurement of LDL-Cholesterol after release of LDL-Cholesterol by detergents in Reagent 2.

Cholesterol ester —(Cholesterol esterase)→ Cholesterol + fatty acid

Cholesterol + O_2 —(Cholesterol esterase)→ Cholestenone + H_20_2

$2H_2O_2$ +4-AA + HDAOS —(Peroxidase)→ Quinoneimine + $4H_20$

The intensity of the quinoneimine dye produced is directly proportional to the cholesterol concentration when measured at 600nm

4-AA = Aminoantipyrine

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

NB: This assay uses a Rate method and a single point calibration.

Appendix V - 25-hydroxyvitamin D (25(OH)D)

25-hydroxyvitamin D (25(OH)D)

Principle

25(OH)D was measured by a commercial kit Liaison® 25(OH)D assay (Diasorin, Italy) to measure serum concentration of 25(OH)D.

The method for quantitative determination of 25(OH)D is a direct competitive chemiluminescence immunoassay. A specific antibody to vitamin D is used for coating magnetic particle (Solid phase) and vitamin D is linked to an isoluminol derivatives. During the incubation the period, 25(OH)D is dissociated from its binding sites on the antibody. After the incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added and a flash chemiluminescence reaction is initiated. The light signal is measured by a photomultiplier as relative light units (RLU) and is inversely proportional to the concentration of 25(OH)D present in calibrators, controls or samples.

In terms of quality assurance, our laboratory participates in an international quality assurance programme (DEQAS - International Vitamin D External Quality Assessment Scheme, London, United Kingdom). Since our participation in DEQAS external quality control, our laboratory has received the certificates of efficiency on a yearly basis (i.e. 80% or more results fell within 30% of the all laboratory trimmed mean). Lipids were measured by a Randox Daytona Instrument (Randox Laboratories Ltd, County Antrim, UK) (intra and inter-assay variation < 5%).

Appendix VI - Dual-energy X-ray absorptiometry

Dual-energy X-ray absorptiometry (DXA)

Principle

The fundamental principle of DXA is the measurement of transmission of x-rays with high- and low-energy photons through the body.

The mathematics used to calculate bone density values can be explained using an exponential equation that assumes the body to be a two-compartment model consisting of bone mineral and soft tissue.

Bone mineral is a physically dense material mainly made up of phosphorus and calcium molecules that have relatively high atomic numbers.

Soft tissue is a mixture of muscle, fat, skin, and water. It has a lower physical density and a lower effective atomic number because its main chemical constituents are hydrogen, carbon, and oxygen.

Appendix VII – Plagiarism declaration

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

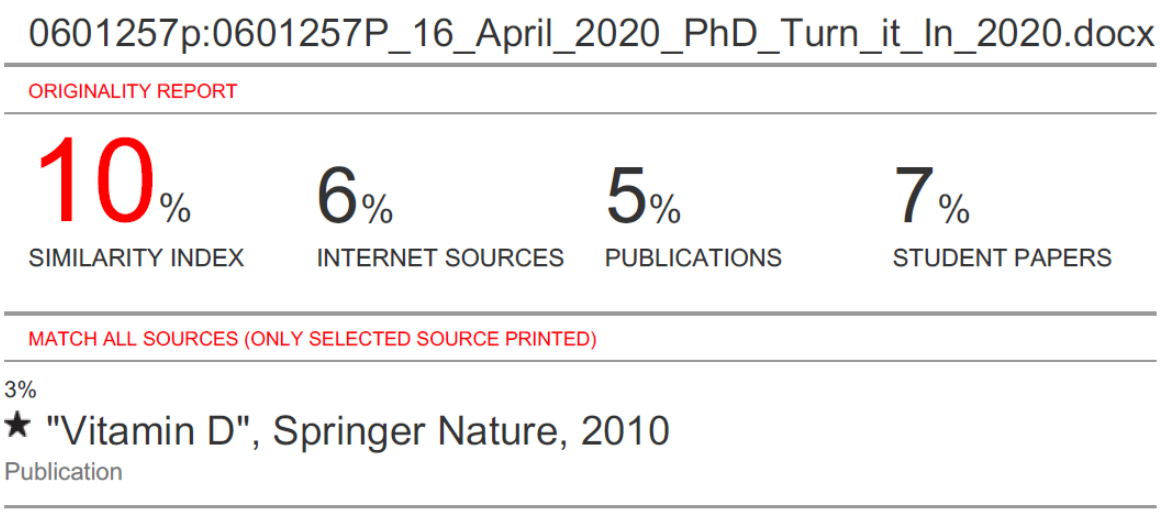
I MACHUENE ANANIAS POOPEDI (Student number: 0601257P) am a student registered for the degree of DOCTOR OF PHILOSOPHY in the academic year 2020.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: _____ Date: 20 April 2020

Appendix VIII-Turn It In Report



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ORIGINALITY REPORT

10%	6%	5%	7%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

MATCH ALL SOURCES (ONLY SELECTED SOURCE PRINTED)

3%

★ "Vitamin D", Springer Nature, 2010

Publication

Appendix IX Ethical clearance I

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Norris

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M980810

PROJECT Longitudinal Study Investigating Bone Health In South African Children

INVESTIGATORS Mr SA Norris

DEPARTMENT Physiology Dept, Wits Medical School

DATE CONSIDERED 980828

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 981201 **CHAIRMAN** .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Prof JM Pettifor
Dept of Paediatrics Dept, Baragwanath Hospital

/Works2/lain0015/HumEth97 wdb/M 980810

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned esearch 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.

DATE 1998-12-04 SIGNATURE .....

PROTOCOL NO.: M 980810

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix X Ethical clearance II

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Poopedi

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M061012**
PROJECT	Calcium Absorption, Homeostasis, and Vitamin D Profiles and their Impact on Bone Mass in Urban Black...
INVESTIGATORS	Mr MA Poopedi
DEPARTMENT	Birth to Twenty Research Prog
DATE CONSIDERED	06.10.27
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

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

DATE 07.01.10 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof JM Pettifor
MACHUENE ANANIAS POOPEDI

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 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