Faculty of Health Sciences
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Vitamin D status and dietary calcium in chronic disease: Potential associations with metabolic syndrome and type 2 diabetes mellitus in Australian adults

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Declaration

To the best of my knowledge and belief, this thesis titled "Vitamin D status and dietary calcium in chronic disease: Potential associations with metabolic syndrome and type 2 diabetes mellitus in Australian adults" contains no material previously published by any other person, except where due acknowledgement has been made. This thesis does not contain material which has been accepted for the award of any other degree or diploma in any university.

The Victorian Health Monitor (VHM) survey was approved by the Human Research Ethics Committee (HREC) of the Baker IDI Heart and Diabetes Institute, Melbourne, Victoria. The analysis of the VHM database was approved by the HREC at Curtin University (HREC approval number: SPH-19-2014). All persons gave their informed consent to their inclusion in the study.

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Abstract

Background: Calcium intake and vitamin D status is poor in Australia despite adequate food sources and sunshine. There is evidence that low calcium intake and low vitamin D status may account for the increased clustering of abdominal obesity and metabolic dysfunction in various population groups worldwide.

Aim: The aim of this thesis was to investigate the associations between vitamin D status, as measured by circulating 25-hydroxyvitamin D (25OHD), and dietary calcium intake to the presence of metabolic syndrome (MetS), its individual components, and the risk of type 2 diabetes mellitus (T2DM) in Australian adults.

Methods: The thesis analysed the Victorian Health Monitor (VHM) survey data which is a population representative sample of 3,409 adults residing in the state of Victoria, aged 18-75 years. Multivariable logistic regression analyses were used to achieve the study objectives, and are described as adjusted odds ratio (AOR) and 95% CI. Complex samples analysis was applied to adjust for the unequal selection probabilities due to the multistage cluster sampling used in VHM. We also conducted a systematic review of the literature and meta-analysis to investigate the association between weight loss in obese subjects and change in vitamin D status.

Results: The mean 25OHD concentration of this population was 56 nmol/L (SD 23.7 nmol/L). The determinants of 25OHD included those who: were obese **(**AOR 0.37, 95% CI 0.29, 0.47), were insufficiently physically active each week (AOR 0.49, 95% CI 0.35, 0.69), were smokers (AOR 0.61, 95% CI 0.39, 0.93), were living in metropolitan areas (AOR 0.53, 95% CI 0.33, 0.87), and had their biomedical examination in winter (AOR 0.10, 95% CI 0.04, 0.29) or spring (AOR 0.11, 95% CI 0.03, 0.40)**.** An additional novel variable was sitting time, where 4-8h per day (AOR 0.63, 95% CI 0.49, 0.80) and ≥8h per day (AOR 0.32, 95% CI 0.22, 0.46) made a measurable difference to 25OHD (Chapter 3).

A systematic review and meta-analysis found that a 10kg loss in body weight may increase plasma 25OHD concentration by around ~6 nmol/L (Chapter 4).

We then investigated the association between: 25OHD concentration, dietary calcium intake and MetS (Chapter 5). Combinations of calcium intake and 25OHD tertiles showed that increasing calcium intake had a protective dose response relationship with MetS up to a median 25OHD status of ~54 nmol/L. At higher values of 25OHD, the effect of calcium was not apparent and only 25OHD had a beneficial effect. A similar analysis for individual components of MetS showed certain combinations of 25OHD and dietary calcium intake had a beneficial effect on elevated triglycerides, and elevated diastolic blood pressure. There was a dose response effect with increasing calcium intake on low high density lipoprotein cholesterol, however at high 25OHD status ~77 nmol/L the effects of calcium were blunted (Chapter 6). Further, we found that higher 25OHD concentration reduced the odds of: elevated fasting plasma glucose (AOR 0.61, 95% CI 0.44, 0.84; p=0.011), elevated HbA1c (AOR 0.74, 95% CI 0.58, 0.93; p=0.041), indicating a potential role in T2DM. These results were obtained even after controlling for components of MetS (Chapter 7).

Conclusion: This thesis confirms the expected determinants of 25OHD but signals that increased sitting time maybe an unrecognised factor. Unlike other investigators, we found that volumetric dilution is not the only reason for a lower 25OHD in obesity. A sequestration effect as well inter-conversion of 25OHD to other metabolites was possible. Overall our analysis implicates both calcium and vitamin D could have a role in the amelioration of individual components of MetS, as well as MetS and T2DM per se. These outcomes at a population level strengthen the epidemiological evidence base in this area and suggest a change to current dogma that calcium and vitamin D are only required for good bone health.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AACE	American Association of Clinical Endocrinologists
ADA	American Diabetes Association
ALTM	All Laboratory Trimmed Mean
AOR	Adjusted odds ratio
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BP	Blood pressure
CATI	Computer assisted telephone interviews
CBA	Competitive protein binding assay
CDs	Census Collection Districts
CLIA	Chemiluminescence immune assays
CVD	Cardiovascular disease
d	Day
DBP	Diastolic blood pressure
DEXA	Dual-energy x-ray absorptiometry
EAR	Estimated average requirement
ECLIA	Electrochemiluminescent immunoassay
EGIR	European Group for the study of Insulin Resistance
EI	Energy intake
ELISA	Enzyme-linked immune absorbent assay
FFM	Fat free mass
FM	Fat mass
FPG	Fasting plasma glucose
GI	Gastrointestinal
h	Hour/s
HbA1c	Glycated haemoglobin
HDL-C	High density lipoprotein cholesterol
HREC	Human Research Ethics Committee
IDF	International Diabetes Federation
IFG	Impaired fasting glucose

IGT	Impaired glucose tolerance
IOM	Institute of Medicine
IR	Insulin resistance
IRSED	Index of relative socioeconomic disadvantage
LC-MS	Liquid chromatography mass spectrometry
LDL-C	Low density lipoprotein cholesterol
MA	Meta-analysis
MetS	Metabolic syndrome
MFS	Modified Fitzpatrick Scale
NCEP: ATP III	National Cholesterol Education Program: Adult Treatment Panel III
nVDR	Nuclear vitamin D receptor
OGTT	Oral glucose tolerance test
OR	Odds ratio
PG	Plasma glucose
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocols
PTH	Parathyroid hormone
RAS	Renin angiotensin system
RCT	Randomised controlled trial
RDI	Recommended dietary intake
REML	Restricted maximum likelihood
RIA	Radioimmunoassay
SBP	Systolic blood pressure
SES	Socio-economic status
SMM	Skeletal muscle mass
SR	Systematic review
TG	Triglycerides
TV	Target Value
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
UV	Ultra violet
UVR	Ultra violet radiation

VDBP	Vitamin D binding protein
VHM	Victorian Health Monitor
VPHS	Victorian Population Health Surveys
vs.	Versus
WC	Waist circumference
WHO	World Health Organization
WHR	Waist:hip ratio
Wks	Weeks
WMD	Weighted mean difference
Y	Years
25OHD	25-hydroxyvitamin D
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
95% CI	95% confidence interval
%FM	Percentage fat mass

Acknowledgements

I would like to acknowledge the School of Public Health, Curtin University for research support. I am a recipient of an Australian Postgraduate Award and Curtin University Postgraduate Scholarship, thus I would like to acknowledge the Office of Research and Development, and the Scholarships office for this opportunity. We would also like to gratefully acknowledge The Victorian Department of Health and Human Services for use of the Victorian Health Monitor survey dataset. I would finally like to acknowledge the great contribution of my supervisor Mario J Soares, and co-supervisors Yun Zhao and Leonard S Piers. The contribution of Zahid Ansari from the Victorian Department of Health is also duly acknowledged.

Dedication

I would like to express my uttermost appreciation and respect for my supervisor Associate Professor Mario J Soares. When I first met you, I was intrigued by your sense of excitement and your need to advance science through research! You continually encouraged me to be a great researcher through writing papers, collaborating with others and reminding me that "It's all about the science". You motivated me to understand the science and think broadly. I am grateful for your continual time, guidance, and wisdom and your true dedication to science, research and your students. You are one of the most insightful and interesting researchers I have ever met, and your knowledge and expertise are admirable.

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grateful. Tanvir, this year marks 11 amazing years together and 5 years married. Our journey together has been nothing but breath taking. However, in these last 3 years you have truly shown me just how loving and thoughtful you are. You have been incredibly supportive, a listening ear and have always been there to provide an encouraging word. I give my deepest expression of love and appreciation to you Tanvir, your warmth and love have provided the greatest support. Thank you for being by my side, and holding my hand all the way. Much love to Jag, Jas and Tanvir.

Chapter 1 Introduction and thesis overview

Introduction

1.1 Vitamin D status

Vitamin D deficiency is a worldwide health issue. Approximately 1 billion individuals, globally, are vitamin D deficient or insufficient (Holick & Chen, 2008). In comparison to other vitamins, the production of vitamin D is unique, as it is synthesised in the skin via exposure to sunlight (Cherniack, Levis, & Troen, 2008). Vitamin D may also be obtained from food, or in the form of supplements. Vitamin D status is determined by circulating serum concentrations of 25-hydroxyvitamin D (25OHD) (Institute of Medicine, 2011). Serum 25OHD is the best indicator of vitamin D status, for a number of reasons. Firstly, 25OHD has a long half-life of ~3 weeks compared to other vitamin D metabolites such as 1, 25 dihydroxyvitamin D ($1,25(OH)_2D$), with a much shorter half-life of ~4 hours (Zerwekh, 2008). Secondly, 25OHD provides an accurate estimate of vitamin D stores from synthesis in the skin and dietary intake, and its production in the liver is not greatly regulated (Zerwekh, 2008). However, the synthesis of other vitamin D metabolites, $1,25(OH)_2D$, are tightly regulated by calcium, parathyroid hormone (PTH) activity and phosphate levels present in the plasma (Rajasree et al., 2001). Lastly, of all the vitamin D metabolites, 25OHD has the highest concentration, thus is the best marker of vitamin D status (Alshahrani & Aljohani, 2013).

The role of vitamin D in the maintenance of bone and muscle health is well established (Institute of Medicine, 2011), and the clinical consequences of low serum vitamin D concentration are rickets (Allgrove, 2009), osteomalacia and osteoporosis (Boonen et al., 2006). However, over the last 20 years, there is a growing recognition that vitamin D has an important role in a number of extra-skeletal functions and vitamin D deficiency may contribute to the development of a number of chronic diseases, such as cancer, multiple sclerosis,

cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), high blood pressure (BP) and the metabolic syndrome (MetS) (Dobnig, 2011; Holick & Chen, 2008). Hence, vitamin D deficiency is an important public health issue, and it affects people of both genders, all ages, and socio-economic backgrounds. Further research that cements its role in this area is urgently required.

1.2 Dietary calcium

Vitamin D and calcium are intimately linked, whereby the key function of vitamin D is to tightly control calcium absorption from food and its homeostasis (Heaney, 2008). Adequate intakes of calcium can be easily achieved through consumption of dairy products. However, despite abundant supplies of milk, calcium intake remain low across the globe (Wang & Li, 2008). Like vitamin D, high intakes of dietary calcium are recommended to improve bone health. However, recent evidence has also implicated calcium in the regulation of body weight (Soares, Murhadi, Kurpad, Chan She Ping-Delfos, & Piers, 2012; Zemel, Shi, Greer, Dirienzo, & Zemel, 2000), and the risk of T2DM (Gijsbers et al., 2016) and MetS (Crichton, Bryan, Buckley, & Murphy, 2011; Dugan & Fernandez, 2014).

1.3 MetS and T2DM

MetS is a constellation of metabolic abnormalities, and an individual is diagnosed as having MetS if they have three of the following markers: visceral adiposity, hypertension, elevated triglycerides (TG), reduced high density lipoprotein cholesterol (HDL-C), and elevated fasting plasma glucose (FPG) (Alberti et al., 2009). An individual is classified as having T2DM based on elevated glycated haemoglobin (HbA1c) or FPG concentrations (American Diabetes Association, 2010). With the prevalence of obesity increasing by 28% in the last 33 years (Ng et al., 2014), it is not surprising that the prevalence of associated diseases, such as MetS and T2DM, are also on the rise (International Diabetes Federation, 2006). MetS criteria were initially established to be able to predict the risk of CVD. However, MetS has been found to increase the risk of other diseases such as non-alcoholic steatohepatitis (Chen

et al., 2011) and T2DM (Ford, Li, & Sattar, 2008). It is well known that lifestyle modifications, including a healthy diet and adequate physical activity are the primary treatment methods for MetS and T2DM. However, given the existence of widespread vitamin D deficiency and low calcium intakes, and increased rates of MetS and T2DM, other potential avenues of treatment should be further investigated.

1.4 Significance: Vitamin D status, dietary calcium intake, and its association with MetS and T2DM

Worldwide, the prevalence of vitamin D insufficiency (<50 nmol/L) is estimated to be between 18% and 36% (Ginde, Liu, & Camargo Jr, 2009; Greene-Finestone et al., 2011; Hypponen & Power, 2006; Langlois, Greene-Finestone, Little, Hidiroglou, & Whiting, 2010; Looker, Dawson-Hughes, Calvo, Gunter, & Sahyoun, 2002; Rockell, Skeaff, Williams, & Green, 2006). However, in a sun soaked country, such as Australia, the prevalence of vitamin D deficiency appears to be slightly higher at around 23% (McGrath, Kimlin, Saha, Eyles, & Parisi, 2001) to 43% (Pasco, Henry, Nicholson, Sanders, & Kotowicz, 2001).Those who are most at risk of vitamin D deficiency are individuals with darker skin pigmentation, veiled women, pregnant women and the elderly (Nowson & Margerison, 2002). Recently, international studies have uncovered other potential determinants of serum vitamin D concentration (Freedman et al., 2013; Lips, van Schoor, & de Jongh, 2014; Touvier et al., 2015). However, very few studies in Australia have investigated the determinants of serum vitamin D concentration (Brodie et al., 2013; Daly et al., 2012; Kimlin et al., 2014), with smaller sample sizes than our study (Brodie et al., 2013; Kimlin et al., 2014) and no inclusion of any dietary variables (Brodie et al., 2013; Daly et al., 2012; Kimlin et al., 2014).

Thus, as a first step in our investigation, we wished to explore the determinants of vitamin D status in Australian adults.

Vitamin D insufficiency and obesity are two current health issues, which are linked with the increase risk of chronic diseases such as MetS and T2DM (Ford et al., 2008). There is

evidence of an inverse relationship between obesity and serum vitamin D concentration, whereby obese individuals tend to have a lower serum vitamin D concentration than their leaner counterparts (Ardawi, Qari, Rouzi, Maimani, & Raddadi, 2011; Beydoun et al., 2010; Brock et al., 2010). It has been suggested that low serum vitamin D concentration may be due to the vitamin being 'sequestered' into greater amounts of adipose tissue (Wortsman, Matsuoka, Chen, Lu, & Holick, 2000), and/or there may be a volumetric dilution effect (Drincic, Armas, Van Diest, & Heaney, 2012). Could weight loss in obese subjects affect vitamin D status? This question had never been posed thus was our next area of interest.

Thus, as a second step in this investigation, we were interested to investigate if weight loss in obese subjects, without supplementary vitamin D, increased serum 25OHD concentration.

Low serum vitamin D concentration and dietary calcium intake have been linked to an increased risk of chronic disease such as MetS (Crichton et al., 2011; Dugan & Fernandez, 2014) and its sequelea T2DM (Gijsbers et al., 2016). Few studies in Australia have explored any association between vitamin D status, MetS and T2DM (Brock et al., 2012; Gagnon et al., 2012; Gagnon et al., 2011; Maple-Brown et al., 2014; Pittas et al., 2006; Pittas, Sun, Manson, Dawson-Hughes, & Hu, 2010b), with only two exploring calcium intake and T2DM (Gagnon et al., 2011; Pittas et al., 2006).

Thus, the final step was to examine the association between serum vitamin D concentration, dietary calcium intake, the presence of MetS, MetS components and the risk of T2DM.

In Figure 1.1, we have presented a diagrammatic overview of the thesis and the topics covered. The dashed red arrows indicate the relationships that have been investigated in the thesis, and the blue boxes indicate the chapter that discusses these topics.

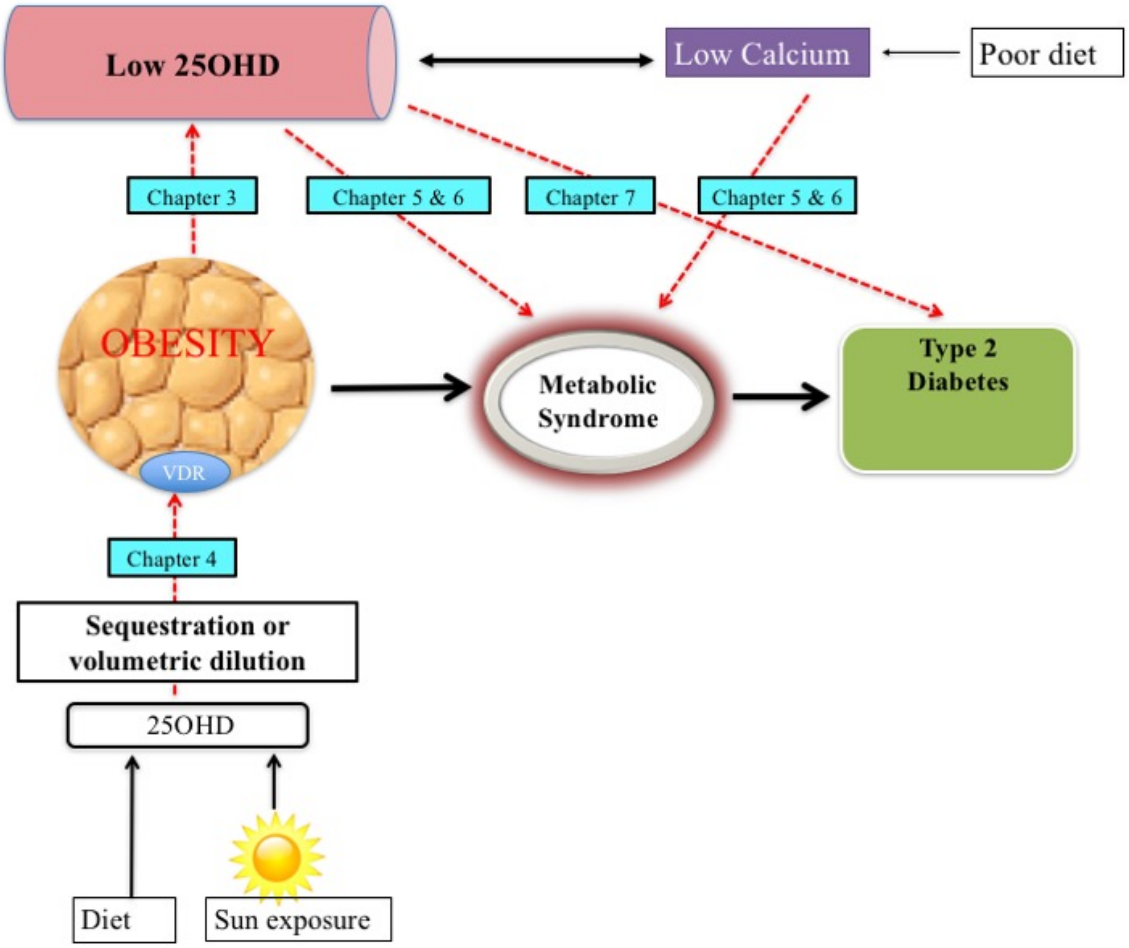

Figure 1.1 Diagramatic overview of thesis and topics covered: possible relationships of serum vitamin D concentration, dietary calcium intake, with health outcomes of obesity, MetS and T2DM.

Footnotes: Blue boxes; indicate the relevant chapters in this thesis, Red arrows; indicate the relationships that have been investigated the thesis.

Thesis overview

1.5 Aim and Objectives

1.5.1 Aim

The overall aim of the thesis was to investigate the associations between vitamin D status and dietary calcium intake with the presence of MetS, and the risk of T2DM by analysing the Victorian Health Monitor (VHM) survey dataset.

1.5.2 Objectives

This thesis has the following specific research objectives:

Objective 1: To review the literature on vitamin D status, dietary calcium, and their association with MetS and T2DM.

Objective 2: To investigate the physical, demographic, and lifestyle determinants of vitamin D status in a population based sample of Australian adults aged 18-75 years.

Objective 3: To verify the role of adiposity and weight loss on vitamin D status.

Objective 4a: To determine if vitamin D status, and/or dietary calcium intake are related to the presence of MetS.

Objective 4b: To determine whether vitamin D status, dietary calcium intake and their combination were associated with individual components of MetS.

Objective 5: To determine if vitamin D status is related to the risk of T2DM.

1.6 Chapter outline and objectives

Chapter	Objective
Chapter 2 Literature review.	**Objective 1** To review the literature on vitamin D status, dietary calcium, and their association with MetS and T2DM.
Chapter 3 The determinants of vitamin D status in Australian adults aged 18-75 years.	**Objective 2** To investigate the physical, demographic, and lifestyle determinants of vitamin D status in a population based sample of Australian adults aged 18-75 years.
Chapter 4 Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects.	**Objective 3** To verify the role of adiposity and weight loss on vitamin D status.
Chapter 5 The associations of vitamin D status and dietary calcium with the metabolic syndrome.	**Objective 4a** To determine if vitamin D status, and/or dietary calcium intake are related to the presence of MetS.
Chapter 6 Vitamin D status and dietary calcium intake and the components of the metabolic syndrome.	**Objective 4b** To determine whether vitamin D status, dietary calcium intake and their combination were associated with individual components of MetS.
Chapter 7 Vitamin D status is inversely associated with markers of risk for type 2 diabetes.	**Objective 5** To determine if vitamin D status is related to the risk of T2DM.
Chapter 8 Summary and conclusions.	

Chapter 2 Literature review[1]

Objective addressed

Objective 1: To review the literature on vitamin D status, dietary calcium intake, and its association with MetS and T2DM.

[1] Sections 2.1.5; 2.5.2; 2.5.3; 2.5.4; 2.7.2; 2.7.3; 2.7.4 of Chapter 2 have be published as a book chapter: Pannu, P. K**.,** Calton, E. K., & Soares, M. J. (2016). Calcium and vitamin D in obesity and related chronic disease. In J. Henry (ed.), *Advances in Food and Nutrition Research*, (Vol. *77)*, chapter 2, page 57-88. London, UK: Academic Press, UK (Appendix A), Reproduced with permission from the publisher (Appendix E) and authors (Appendix F).

2.1 Vitamin D

2.1.1 The history of vitamin D

Vitamin D, also known as calciferol, was referred to as "D" and was the fourth identified vitamin (Pilz et al., 2013), The role of this fat soluble vitamin was first described in the prevention and cure of rickets (Chick, Palzell, & Hume, 1923; McCollum, Simmonds, Becker, & Shipley, 1922). Vitamin D deficiency was hence linked to impaired skeletal development, mainly found in children, or osteomalacia in adults. Early studies found that those consuming cod liver oil, those exposed to sunlight, or ultraviolet (UV) light, were able to avoid the development of rickets (Chick et al., 1923; McCollum et al., 1922). This lead to the discovery that vitamin D was able to cure not only rickets, but also muscular spasms referred to as tetany (DeLuca, 2011). One of the other earliest discovered roles of vitamin D was its function in the homeostasis, absorption and utilisation of calcium (Kletzien, Templin, Steenbock, & Thomas, 1932; Nicolaysen, 1937). Kletzien et al. (1932) and Nicolaysen (1937) found that vitamin D was essential in order to utilise and absorb dietary calcium. Over time it was confirmed that vitamin D was essential in absorbing calcium from the gut in times of need, establishing its role in bone health.

It was soon discovered that vitamin D could offer more than aiding good bone health. Vitamin D receptors were identified in a number of tissues including the kidney, parathyroid gland, islet cells of the pancreas and selected neural cells (Stumpf, Sar, Reid, Tanaka, & DeLuca, 1979). This suggested other potential roles of vitamin D. Since then, studies have uncovered associations between low serum vitamin D and an increased risk of hypertension, heart disease, MetS and T2DM (Nair & Maseeh, 2012; Theodoratou, Tzoulaki, Zgaga, & Ioannidis, 2014). With the increasing prevalence of obesity and associated diseases, such as MetS and T2DM, there is a sense of urgency to investigate the associations between vitamin D status and chronic disease.

2.1.2 Vitamin D metabolism

Vitamin D is obtained from either synthesis in the skin via sun exposure, or from the diet. Once in the blood stream, vitamin D is transported by the vitamin D-binding protein (Ralph, Lucas, & Norval, 2013) (Figure 2.1). Two hydroxylation steps convert the inactive vitamin D to its active metabolite, $1,25(OH)_2D$ (calcitriol) (Ralph et al., 2013). The first step occurs in the liver whereby vitamin D undergoes 25-hydroxylation to form 25OHD (Ralph et al., 2013). The enzyme involved in the hydroxylation is CYP2R1, and/or CYP27A1 (Lehmann, Querings, & Reichrath, 2010). Then, the enzyme 1-alpha hydroxylase (CYP27B1) catalyses the conversion of 25OHD to $1,25(OH)_2D$ in the mitochondria of the kidney (Ralph et al., 2013). It is recognised that this enzyme is present not only in the kidneys, but also in the pancreas (Pilz et al., 2013) and immune cells including T cells, B cells, and macrophages (Bouillon et al., 2014; Hossein-Nezhad et al., 2013; Neve, Corrado, & Cantatore, 2013). Thus several cell types have the ability to locally produce $1,25(OH)_2D$ from 25OHD. The active metabolite can then bind to the nuclear vitamin D receptor (nVDR), where it forms a heterodimer with the retinoid X receptor (Pilz et al., 2013). This complex binds to certain DNA regions, called vitamin D responsive elements. Recruitment of coactivators and enzymes with histone acetylation activity results in structural changes in chromatin and facilitates gene transcription (Zhang et al., 2012). In fact, the VDR regulates approximately 3% of the human genome (~700 genes) (Pertea & Salzberg, 2010; Pilz et al., 2013). Nuclear VDRs are present in the majority of cells of the body and forms the basis for the investigations into extra-skeletal benefits of vitamin D. Table 2.1 indicates the terms used in reference to vitamin D.

Table 2.1 The terms used in reference to vitamin D.

Molecular name	Molecular formula	Other terms
25OHD	25-hydroxyvitamin D	Calcidiol *or* Calcifediol
$1,25(OH)_2D$	1,25-dihydroxyvitamin D	Calcitriol
Vitamin D= vitamin D_2 +vitamin D_3		Vitamin D *or* Calciferol
$25OHD_2$	25-hydroxyvitamin D_2	Vitamin D_2 *or* Ergocalciferol

Molecular name	Molecular formula	Other terms
$25OHD_3$	25-hydroxyvitamin D_3	Vitamin D_3 *or* Cholecalciferol

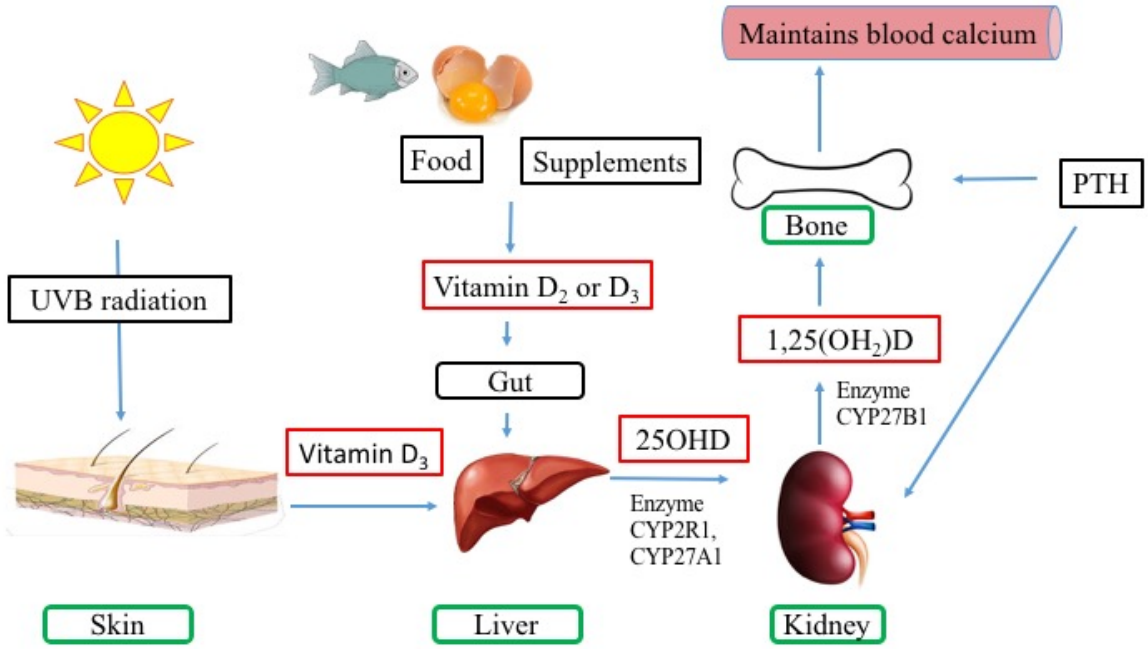

Figure 2.1 The metabolism of vitamin D in the body.

Footnotes: Red box; denotes the form of vitamin D, Green box; denotes the body part that is responsible for the conversion of vitamin D to its metabolites.

2.1.3 Sources of vitamin D

The three key sources of vitamin D are: cutaneous synthesis, dietary intake (naturally occurring or fortified), and supplements. The two forms of vitamin D in the diet are; vitamin D_2 (ergocalciferol), and vitamin D_3 (cholecalciferol) (Holick, 2006a). Vitamin D_2 is synthesised through the irradiation of the sterol, ergosterol, which is only found in fungi, such as mushrooms, and yeast. Vitamin D_3 is naturally present in certain foods, and is also synthesised in the skin after UV exposure (Holick, 2006a).

The primary source of serum vitamin D is from endogenous synthesis in the skin (Pilz et al., 2013). Skin that is exposed to either sunlight, or artificial sources of UVB, is the major

source of vitamin D_3. Approximately 90% of vitamin D_3 is from synthesis in the skin, with the remainder from diet (Lehmann et al., 2010).

For most populations, diet is the smallest contributor to vitamin D stores (Webb, Pilbeam, Hanafin, & Holick, 1990). Vitamin D occurs naturally in some foods, mainly fatty fish such as salmon, sardines, tuna, mackerel, herring and cod (Nowson & Margerison, 2002), as well as shitake mushrooms and eggs. In Australia, it may also be fortified in some foods including butter, margarines and some cereals (Oberhelman & Thacher, 2013). Mushrooms are a natural source of vitamin D, and more recently mushrooms in Australia which were exposed to UV light, were available as vitamin D enriched mushrooms (Food Standards Australia New Zealand, 2007). With limited foods fortified with vitamin D in Australia, and natural food sources containing small amounts of the vitamin, food sources are not a significant contributor to vitamin D status (Webb et al., 1990).

More recently dietary supplement usage has become more widespread and is another important source of vitamin D (Bikle, 2010). However, in Australia where around a quarter of the population are vitamin D deficient (Australian Bureau of Statistics, 2013a), only ~5% of the population were taking vitamin D supplements (Black, Jacoby, Nowson, Daly, & Lucas, 2016). In Australia, females, older age groups, a higher socio-economic/educational status, non-smokers and physically active people are more likely to consume vitamin D containing supplements (Black et al., 2016). Due to the low levels of supplement intake for a variety of reasons, encouraging appropriate and safe levels of sun exposure may assist in reducing the rates of vitamin D deficiency.

2.1.4 Causes of vitamin D deficiency

Approximately 50% of the world's population has been classified as being vitamin D insufficient based on a 25OHD value of 50 nmol/L (Holick & Chen, 2008; Lips, 2010), regardless of ethnicity, age or geographical location. Vitamin D status and season are intrinsically linked. Season has often been used as a proxy measure for vitamin D status, as

~90% of circulating vitamin D (Holick, 2004) is from the synthesis in the skin after sunlight exposure (Holick et al., 2011). There appears to be a sinusoidal pattern between vitamin D status and month, with the highest concentrations in summer and autumn, and the lowest concentrations in winter (Kasahara, Singh, & Noymer, 2013). In Australia, approximately 31% of adults have insufficient levels of vitamin D, with this drastically increasing to 50% in females during the seasons of winter and spring in the southern parts of the country (Nowson & Margerison, 2002). There are a number of potential reasons for high prevalence of vitamin D deficiency (Table 2.2) such as a reduced skin synthesis due to limited sun exposure, darker skin pigmentation, sun seeking behaviour, and seasonal variation. There are also certain conditions, as listed in Table 2.2, which may decrease the bioavailability, reduce the synthesis or increase the urinary loss of 25OHD. Aside from health conditions, the general characteristics of those who are at risk of vitamin D deficiency are those who tend to spend more time indoors due to work or live in residential care, have darker skin, avoid sun exposure or have increased adipose tissue (Nowson & Margerison, 2002). Thus, these commonly occurring factors, may affect the vast proportion of the population.

Table 2.2 Causes of vitamin D deficiency.

Causes	Reason
Reduced production of vitamin D in the skin	Darker skin pigmentation. Seasonal variation/latitude/time of day. Aging Those with skin grafts.
Breastfeeding	Increase risk of vitamin D deficiency in infants who are exclusively breastfed as breast milk is a poor source of vitamin D.
Decreased bioavailability	Diseases causing malabsorption: cystic fibrosis, celiac disease, Whipple's disease, Crohn's disease, bypass surgery, medications that reduce cholesterol absorption. Obesity
Increased catabolism	Medications: anticonvulsants, glucocorticoids, AIDS treatment, antirejection drugs.
Reduced synthesis of 25OHD	Liver failure.

Causes	Reason
Increased urinary loss of 25OHD	Nephrotic syndrome.
Reduce synthesis of $1,25(OH)_2D$	Chronic renal failure.
Hereditary	Genetic mutations resulting in rickets.
Acquired disorders	Tumour-induced osteomalacia, primary hyperparathyroidism, hyperthyroidism and other conditions.

(Holick, 2007)

2.1.5 Vitamin D status

Individuals are assessed for their *vitamin D status* by measurement of circulating *25OHD* concentration in the serum (Aloia et al., 2008; Cherniack et al., 2008; Martini & Wood, 2006). 25OHD is derived from vitamin D intake via the diet and from synthesis in the skin (Holick, 2006a, 2006b). There is a lack of agreement on the ideal cut-offs for vitamin D between countries, as well as the interchangeable use of the terms 'deficiency', 'sufficiency', 'insufficiency', 'adequacy' or 'inadequate'. The lack of consensus around cut-offs and terminology makes it difficult to compare prevalence (Spiro & Buttriss, 2014). Currently, the evidence based review of the Institute of Medicine (IOM) (Institute of Medicine, 2011) is one of the more widely used cut-offs for 25OHD, as per Table 2.3 below. The IOM has indicated that 50 nmol/L is an optimal level of 25OHD for good bone health for most of the population (Institute of Medicine, 2011). However, some experts have questioned this cut-off, arguing that it may be inadequate for other health outcomes such as hypertension and diabetes (Heaney, 2008; Peterlik & Cross, 2005). An amount of ~75 nmol/L has been suggested as the optimal level for a number of health outcomes (Bischoff-Ferrari, Giovannucci, Willett, Dietrich, & Dawson-Hughes, 2006; Heaney, Horst, Cullen, & Armas, 2009; Norman & Bouillon, 2010).

Table 2.3 Institute of Medicine recommendations for 25OHD concentration for good bone health.

	25OHD (nmol/L)	25OHD (ng/ml)
Sufficient	50	20

	25OHD (nmol/L)	25OHD (ng/ml)
Insufficient	20-50	12-20
Mild deficiency	20-30	8-12
Severe deficiency	<20	<8

(Institute of Medicine, 2011)

Footnote: 1 ng/ml = 2.5 nmol/L

2.1.6 Measurement of serum vitamin D concentration

Serum vitamin D concentration is measured in a laboratory through use of a reliable assay. The half-life of 25OHD is estimated to be ~3 weeks (Holick, 2006a, 2006b), which is considerably longer than other vitamin D metabolites (Zerwekh, 2008). The more commonly used measures of 25OHD are competitive protein binding assay (Haddad & Chyu, 1971), high-performance liquid chromatography (Eisman, Shepard, & DeLuca, 1977), enzyme immunoassay (Lind, Chen, & Byrjalsen, 1997), radioimmunoassay (Hollis & Napoli, 1985) or assays based on liquid chromatography-tandem mass spectrometry (LC-MS) (Maunsell, Wright, & Rainbow, 2005); the latter being the current gold standard. However, the accuracy of serum vitamin D concentration may vary according to the assay used and the laboratory analysing the sample (Black et al., 2015) . When comparing a laboratory using LC-MS against a certified laboratory, vitamin D concentration was 12.4-12.8 nmol/L higher in the uncertified laboratory (Black et al., 2015). In an earlier study it was found that serum vitamin D concentration may differ by up to two-fold between laboratories using the same sample and same technique (Binkley et al., 2004). It is therefore important that laboratories should comply with quality assurance systems to minimise differences in the measurement of 25OHD (Black et al., 2015). The immediate implication of differences in assays used, is the prevalence estimates of vitamin D deficiency in various countries will be different even for the same cut-offs used, and this makes global comparisons difficult.

2.2 Dietary calcium intake

2.2.1 Calcium in the body

Approximately 1-2% of body weight is calcium, making it the most abundant mineral that is physiologically present (Nordin, 1997). Bone stores the majority of calcium in the body, from where it is mobilised in times of metabolic need (Institute of Medicine, 2011). Around 99% of calcium is found in bones and teeth, as calcium hydroxyapatite providing the tissue with strength (Institute of Medicine, 2011). The remaining 1% is present in blood, extracellular fluid, muscles and other tissues, and is involved in vascular contraction and vasodilation, muscle function, nerve transmission, intracellular signalling and hormonal secretion (Institute of Medicine, 2011).

2.2.2 Sources of dietary calcium

The richest food source of calcium is milk and milk-based products including, yoghurt and cheese (National Health and Medical Research Council, 2013). Other food sources include fish with bones, tofu, legumes, some nuts, fortified soy based products and breakfast cereals (National Health and Medical Research Council, 2013). The Australian Dietary Guidelines recommend at least two to three serves of dairy per day which, equates combination of 250ml milk or milk alternatives (soy, rice or other cereal drink), 40g of cheese or 200g of yoghurt (National Health and Medical Research Council, 2013). In Australia, the primary source of calcium intake comes from milk (62%), cheese (29%), yoghurt and dairy snacks eg. custard and fromaige frais (7.9%), and <2% from dairy alternatives (Australian Bureau of Statistics, 2013b). Milk is consumed mainly in beverages, and secondly in cereals (Australian Bureau of Statistics, 2013b).

2.2.3 Calcium intakes worldwide

Between 1980 and 2003, the total dairy production worldwide increased from 475 million to 626 million metric tons (Wang & Li, 2008). This however is not reflected in an increase in

the proportion of the global population with adequate intakes of calcium. There is no global consensus on the recommended daily intakes for calcium. The recommended intakes for calcium in Australia for adults aged 18 years and above are stated in Table 2.4.

There is limited data on global calcium intakes, which makes it difficult to ascertain the proportion of the population that is meeting the required intakes. However, one study analysed the worldwide trends in production and consumption of dairy products and calcium intake in the United States of America (USA), Russia, China and India, which accounts for over half the world's population (Wang & Li, 2008). This study found that none of the countries were meeting the recommended intake of calcium. Those in the USA were not meeting the recommended 1000 mg/d of calcium intake, with male's intake at 962 mg/d and female's intake at 756 mg/d. Those in China were far below the recommended intake of 1000 mg/d, having intakes of 389 mg/d, with around only 10% consuming the required amounts of calcium. The data from Russia is limited, though a study in Moscow found that 70% of participants did not meet the recommended intakes of calcium. India was also well below the recommended intake of 1000 mg/d, with the population consuming around 453 mg/d. It is important to note that the major source of calcium in the Western diet is from dairy products. However, in countries such as China and India, vegetables, beans and bean products, wheat and rice are the major sources of calcium. Calcium present in plant based sources are less bioavailable than dairy products, thus the uptake of calcium in the body is limited (Wang & Li, 2008).

2.2.4 Calcium intakes in Australia

In Australia, the required intake of nutrients are described as a Recommended Dietary Intake (RDI), and an Estimated Average Requirement (EAR). The RDI is the described as the daily dietary intake that is sufficient to meet the requirements of 97-98% of healthy individuals. The EAR is the estimated daily nutrient level required to meet the requirements of 50% of healthy individuals (National Health and Medical Research Council, 2006). The

most recent data from the Australian Health Survey 2011-12 found that more than half of Australians aged two years and above had below adequate intakes of calcium from food sources. Around 73% of males and 51% of females aged two years and above, did not consume the recommend amount of calcium (Table 2.4). Approximately 44% of males and 71% of females aged 19-30 years, did not meet the EAR for calcium. As adults got older, this statistic appeared to gradually increase with 63% of males and 91% of females aged 51-70 years having inadequate calcium intakes. Overall, approximately 10% of Australians met the required dairy intake of two to three serves per day (Australian Bureau of Statistics, 2013b).

Table 2.4 The RDI and EAR for Australian adults according to the Nutrient Reference Values for Australia and New Zealand.

Age (y)	Gender	RDI (mg/d)	EAR (mg/d)
18	Males & Females	1,300	1,050
19-50	Males & Females	1,000	840
51-70	Males	1,000	840
51-70	Females	1,300	1,100
>70	Males & Females	1,300	1,100

(National Health and Medical Research Council, 2006)

Footnotes: d, day; EAR, estimated average intake; RDI, recommended dietary intake; y, years.

2.2.5 Role of calcium in the body

Serum calcium is tightly controlled in the body, so that plasma Ca is maintained within a tight range of 8.9-10.1 mg/dl (2.2-2.5 mmol/L) (Blaine, Chonchol, & Levi, 2015).This is accomplished by close relationships between absorption, storage, secretion and excretion of ionised calcium (Moe, 2008). The interplay between vitamin D and calcium are crucial for physiological functions. The PTH-vitamin D endocrine system, tightly regulates calcium metabolism (Institute of Medicine, 2011). When plasma calcium levels are low, PTH and $1,25(OH)_2D$ are secreted until calcium levels are brought back to the normal range (Moe, 2008).

2.2.6 Calcium and vitamin D metabolism

The actions of calcium and vitamin D in the body are tightly linked. One key function of vitamin D is to modulate calcium and phosphorous homeostasis (Cherniack et al., 2008; Pittas, Lau, Hu, & Dawson-Hughes, 2007). During times of vitamin D deficiency, intestinal calcium absorption is reduced and ionized calcium is temporarily lowered. This triggers the calcium sensor in the PTH glands to secrete more PTH (Brown et al., 1993). PTH modulates calcium metabolism in the body via increasing reabsorption of calcium in the kidney, increasing mobilization of calcium from the skeleton and increasing conversion of 25OHD to $1,25(OH)_2D$ or calcitrol in the kidneys (Holick, 2006a, 2006b; Holick & Chen, 2008; Hollis, 2008).

2.3 Obesity

2.3.1 Definition of obesity

Obesity can be defined as an excessive accumulation of fat in adipose tissue (World Health Organization, 2000). Body mass index (BMI) has been commonly used to classify an individual's weight status as underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (≥25-<30 kg/m^2) and obese (≥30 kg/m^2) (Lobstein, 2015; Muller et al., 2012). BMI is calculated as body weight (kg) divided by height squared (m) (Hawkesworth, 2013). Body weight however includes both lean and fat mass, thus there is potential for misclassification of individuals with higher lean mass (Sorensen, Virtue, & Vidal-Puig, 2010). Furthermore, BMI does not reflect fat distribution, thus is a crude estimation for obesity (Song et al., 2013). In addition, individuals may be classified as 'normal weight' using BMI, however can still be insulin resistant and have a high risk of cardio-metabolic disorders; thus analysis of body composition based on valid standards are important (Muller et al., 2012).

Fat distribution, particularly abdominal obesity is strongly associated with T2DM, CVD (Roriz et al., 2014) and MetS (Goodpaster et al., 2005). Obese individuals with MetS tend to have more abdominal visceral fat and less thigh subcutaneous fat, than those without MetS

(Koster et al., 2010). Clinical measures such as dual energy X-ray absorptiometry (DEXA) are the gold standard in quantifying fat distribution, including visceral obesity. Measurement of waist circumference (WC) infers the excess accumulation of visceral adipose tissue (Dobbelsteyn, Joffres, MacLean, Flowerdew, & The Canadian Heart Health Surveys Research Group, 2001). WC is generally used as a surrogate indicator of visceral adipose tissue (Oka et al., 2009). Other measures of adiposity that are strong indicators of visceral obesity include waist-to-hip ratio (WHR), and waist-to-height ratio (Lobstein, 2015; Song et al., 2013). Studies have indicated that these measures are preferred to BMI, as they are strong indicators of abdominal adiposity and metabolic risk factors (Browning, Hsieh, & Ashwell, 2010; Lemieux, Prud'homme, Bouchard, Tremblay, & Despres, 1996; Pouliot et al., 1994; Rankinen, Kim, Perusse, Despres, & Bouchard, 1999).

2.3.2 Prevalence of obesity

The most recent data from the systematic analysis for the Global Burden of Disease study 2013, found that obesity has increased by ~28% over the last 33 years. In Australia, the most recent National Health Survey found that 63% of adults were classified as being overweight or obese with a 56% increase since 1995 (Australian Bureau of Statistics, 2015). The high proportion of obese individuals is a health issue in itself, however it also increases the risk of other associated diseases.

The health outcomes attributed to obesity are extensive. A large body of evidence has consistently linked higher BMI with CVD, cancer, diabetes, osteoarthritis and other chronic diseases (Ni Mhurchu et al., 2004; Prospective Studies Collaboration, 2009; Renehan, Tyson, Egger, Heller, & Zwahlen, 2008). Recent Australian evidence has indicated the prevalence of a number of long term health conditions that tend to congregate with obesity including: high cholesterol (7.1%), hypertension (11.3%), diabetes (5.1%) and heart disease (5.2%) (Australian Bureau of Statistics, 2015). Evidence from a large pooled analysis indicated that high BP, cholesterol and glucose contributed to more than half the risk of coronary heart

disease due to high BMI (The Global Burden of Metabolic Risk Factors for Chronic Diseases Collaboration, 2014). Adiposity appears to be a causal factor of these three metabolic risk factors, which are also key features of MetS. This indicates the public health importance of dealing with obesity and its related risk factors, which present as MetS.

2.3.3 Vitamin D concentration and obesity

Vitamin D is a fat soluble vitamin, and its storage and release from adipose tissue is still not completely understood. Adipose tissue is the major store, due to the hydrophobic nature of the vitamin. This was first proposed by Rosenstreich, Rich, and Volwiler (1971) who investigated the accumulation of vitamin D in vitamin D deficient rats. The rats were given radio-labelled vitamin D and it was found that on repletion of vitamin D, adipose tissue acquired the highest proportion of the compound as well as showed the slowest release rate. Liel, Ulmer, Shary, Hollis, and Bell (1988) found a similar occurrence in humans, wherein obese subjects tended to have lower 25OHD levels than their lean counterparts, due to a potential increased clearance and enhanced uptake of the vitamin into adipose tissue. Wortsman et al. (2000) then proposed that vitamin D was sequestered in adipose tissue and was not released. Drincic et al. (2012) countered this finding by concluding that volumetric dilution per se explained the low vitamin D status in obesity due to the larger body mass and a dilution effect. Clearly, there are two facets to vitamin D metabolism as it pertains to increasing adiposity and hence obesity. One is whether 25OHD is ‘sequestered’ in adipose tissue, or secondly, whether there is a volumetric dilution effect. It is also feasible to include a bit of both of these phenomenon when studying vitamin D status and its links to obesity.

Over the last five years there have been a number of studies that have investigated the associations between vitamin D status and different measures of adiposity from anthropometric profiles to more precise measures of body fat distribution and its quantification (Table 2.5). A mendelian randomisation analysis identified a potential causal association between BMI and 25OHD, where every 10% increase in BMI was associated with a decrease

in 25OHD concentration by 4.2% (Vimaleswaran et al., 2013), with no evidence of the reverse association. Two systematic reviews (SR) and meta-analysis (MA) also found a similar association between BMI and 25OHD (Pereira-Santos, Costa, Assis, Santos, & Santos, 2015; Saneei, Salehi-Abargouei, & Esmaillzadeh, 2013), with a 35% greater prevalence of vitamin D deficiency in those who were obese compared to healthy individuals (Pereira-Santos et al., 2015). However the association was weak due to lack of control for confounders such as physical activity and dietary intakes (Saneei et al., 2013). A combination of cohort and cross-sectional studies found an inverse association between BMI and 25OHD (Bellone et al., 2014; Gonzalez, Ramos-Trautmann, Diaz-Luquis, Perez, & Palacios, 2015; Jungert, Roth, & Neuhäuser-Berthold, 2012; Mai, Chen, Camargo, & Langhammer, 2012; Samuel & Borrell, 2013; Shinkov et al., 2015; Turer, Lin, & Flores, 2013), with others finding no association (Baradaran, Behradmanesh, & Nasri, 2012; Gonzalez et al., 2015; Han et al., 2014; Sulistyoningrum, Green, Lear, & Devlin, 2012). Others found an inverse association between hip circumference (Bellone et al., 2014; Jungert et al., 2012), WC (Bellone et al., 2014; Gonzalez et al., 2015; Mai et al., 2012), WHR (Gonzalez et al., 2015), % body fat (Jungert et al., 2012; Shantavasinkul et al., 2015; Vitezova et al., 2016); visceral adipose tissue (Sulistyoningrum et al., 2012), and android:gynoid fat ratio (Andreozzi et al., 2016). Only one study included fat free mass (FFM) as an outcome, and found a positive correlation between 25OHD and skeletal muscle mass (SMM) (Shantavasinkul et al., 2015). However, the variation in study details such as the sample size, age group, latitude of country, variability in vitamin D status due to the assays used, and variances in tools for obesity measures ie bioelectrical impedance analysis (BIA) vs. DEXA make comparability of results difficult. The measures of obesity were varied thus a conclusive finding was difficult to ascertain. When coupled together, vitamin D deficiency and obesity may further increase the risk of chronic diseases as compared to its independent effects. Thus, understanding the factors that may compromise sufficient vitamin D status is important. Moreover, investigating whether weight loss may benefit vitamin D status would be of interest.

Table 2.5 Studies showing an inverse association between vitamin D status and a measure of obesity.

Authors and year of publication	Study details	BMI / A:G fat ratio	WC / HC / WHR	FM / %FM / AT / SAT / VAT	FFM / SMM
Baradaran et al. (2012)	Age: 20-64 y Subjects: n=259 (110 M,149 F) Study type: Not stated Location: Iran	BMI: No			
Jungert et al. (2012)	Age: 66-96 y Subjects: n=131 (41 M, 90 F) Study type: Cross-sectional Location: Germany	BMI: Yes (women only)	HC: Yes (women only) WC: No WHR: No	FM: Yes (women only)	FFM: No
Mai et al. (2012)	Age: 19-55 y Subjects: n=2,460 (M, F) Study type: Cohort Location: Norway	BMI: Yes BW: Yes	WC: Yes		
Sulistyoningrum et al. (2012)	Age: mean 44-50 y Subjects: n=379 (195 M, 184 F) Study type: Cross-sectional Location: Canada	BMI: No	WC: No	%FM: No AT: No SAT: No VAT: Yes	
Gonzalez et al. (2015)	Age: mean 53.7 y Subjects: n=797 (291 M, 506 F) Study type: Cross-sectional Location: Puerto Rico	BMI: Yes	WC: Yes		

Authors and year of publication	Study details	BMI / A:G fat ratio	WC / HC / WHR	FM / %FM / AT / SAT / VAT	FFM / SMM
Samuel and Borrell (2013)	Age: >18 y Subjects: n=12,927 (M, F NA) Study type: Cross-sectional Location: USA	BMI: Yes			
Saneei et al. (2013)	Age: >18 y Subjects: n=34 articles (n not stated, M, F) Study type: SR and MA from inception-2012 Location: Not stated	BMI: Yes			
Turer et al. (2013)	Age: 6-18 y Subjects: n=12,292 (M, F) Study type: Cross-sectional Location: USA	BMI: Yes			
Vimaleswaran et al. (2013)	Age: mean 31.1–74.9 y Subjects: n=42,024 (M, F) Study type: Mendelian randomisation analysis Location: Europe, USA, Canada.	BMI: Yes			
Bellone et al. (2014)	Age: mean 11.2 y Subjects: n=557 (287 M, 270 F) Study type: Cross-sectional	BMI: Yes	WC: Yes HC: Yes		

Authors and year of publication	Study details	BMI / A:G fat ratio	WC / HC / WHR	FM / %FM / AT / SAT / VAT	FFM / SMM
	Location: Italy				
Han et al. (2014)	Age: mean 59.7 y Subjects: n=1,697 (743 M, 954 F) Study type: Cross-sectional Location: Korea	BMI: No	WC: No	FM: Yes	
Gonzalez et al. (2015)	Age: mean 53.7 y Subjects: n=797 (291 M, 506 F) Study type: Retrospective Location: Puerto Rico	BMI: Yes	WC: Yes WHR: Yes		
Pereira-Santos et al. (2015)	Study type: SR and MA from inception to 2014 of observational studies	BMI: Yes			
Shantavasinkul et al. (2015)	Age:15-70 y Subjects: n=163 (66 M, 97 F) Study type: Cross-sectional Location: Thailand			%BF: Yes	SMM: Yes (positive correlation)
Shinkov et al. (2015)	Age: mean 47.2 y (M), 49.5 y (F) Subjects: n=1,952 (915 M, 1,027 F) Study type: Cross-sectional Location: Bulgaria	BMI: Yes (F only)			
Andreozzi et al. (2016)	Age: mean 64.4 y Subjects: n=62 (F)	A:G fat ratio: Yes	WC: Yes		

Authors and year of publication	Study details	BMI / A:G fat ratio	WC / HC / WHR	FM / %FM / AT / SAT / VAT	FFM / SMM
	Study type: Cross-sectional Location: Italy				
Vitezova et al. (2016)	Age: ≥55 y Subjects: n=2,158 (931 M, 1,227 F) Study type: Prospective Location: Netherlands	A:G fat ratio: No		%FM: Yes	

Footnotes: %FM, % fat mass; A:G fat ratio, android:gynoid fat ratio; AT, adipose tissue; BMI, body mass index; BW, body weight; FFM, fat free mass; FM, fat mass; F, female; HC, hip circumference; M, male; SAT, subcutaneous adipose tissue; SMM, skeletal muscle mass; VAT, visceral adipose tissue; WC, waist circumference; WHR, waist:hip ratio.

2.4 The gap

The inverse relationship between vitamin D status and obesity is quite consistently observed. What is not clear is whether these are cause and effect. Potential explanations may be that the fat soluble vitamin may be 'sequestered' in adipose tissue, or there may be a volumetric dilution effect at play. If the former, then the potential effect of weight loss on vitamin D status should be minimal since the term sequestration suggests something tightly held or unavailable to the system. In contrast, a simple volumetric dilution phenomenon should allow the release of 25OHD back into circulation when adipose tissue mass is reduced. The question hence arose whether targeted weight loss in obese individuals would help improve their vitamin D status?

2.5 MetS

2.5.1 Overview of MetS

Also referred to as 'syndrome X', 'the deadly quartet' (Kaplan, 1989) and the 'insulin resistance syndrome', MetS has been described in various forms since Kylin (1923) in the 1920's. Kylin (1923) noticed that hypertension, hyperglycemia and gout tend to cluster together. In 1947, Vague (1947) identified that visceral obesity was as an additional factor associated with metabolic abnormalities. However, its definition became firmly established after the 1988 Banting lecture by Reaven (1988a). Reaven (1988a) noticed that multiple risk factors for T2DM and CVD, including dyslipidemia, hypertension, and hyperglycemia, tend to occur together and referred to this as 'syndrome x'. Reaven's key addition to the previous descriptions of MetS was the inclusion of insulin resistance (IR) as part of the disease state (Reaven, 1988a). In 1989, Kaplan (1989) grouped together the aforementioned components of obesity, hyperglycemia, hypertriglyceridemia and hypertension and called it 'the insulin resistance syndrome'. Thus, MetS consists of the clustering of factors including elevated WC,

reduced HDL-C, elevated TG, elevated FPG and elevated systolic BP (SBP)/diastolic BP (DBP) (Figure 2.3). At this stage, MetS became an accepted term however there was a need for a universally accepted definition.

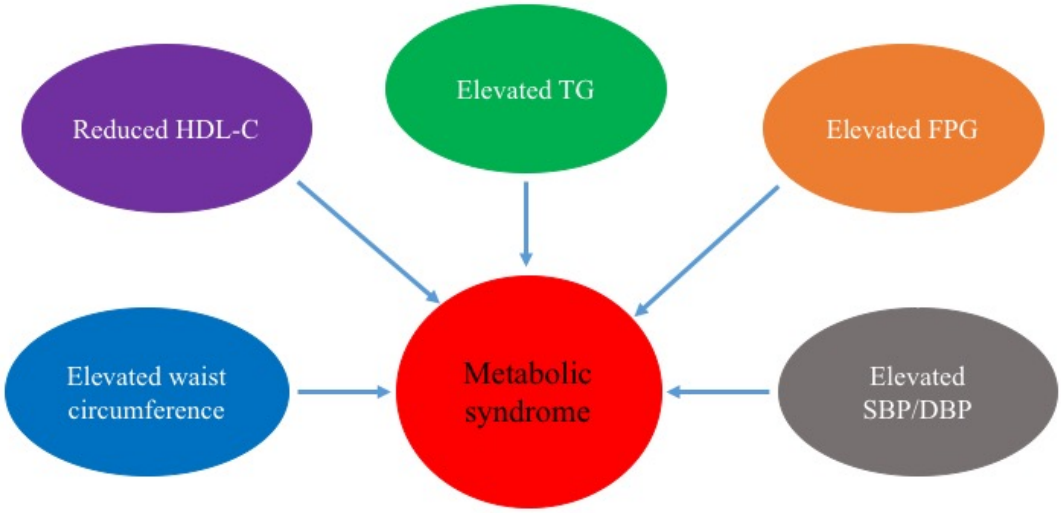

Figure 2.2 Components of the metabolic syndrome.

Footnotes: DBP; diastolic blood pressure, FPG; fasting plasma glucose, HDL-C; high density lipoprotein cholesterol, SBP; systolic blood pressure, TG; triglycerides.

2.5.2 Current definitions of MetS[1]

In 1998 the World Health Organization (WHO) (Alberti & Zimmet, 1998) developed a set of criteria, and this was closely followed by the European Group for the Study of Insulin Resistance (EGIR) (Balkau & Charles, 1999), the National Cholesterol Education Program's Adult Treatment Panel II (NCEP: ATP III) (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001) and the American Association of Clinical Endocrinologists (AACE) (Einhorn et al., 2003). In order to provide a single definition, the International Diabetes Federation (IDF) proposed the most recent definition in 2005 (International Diabetes Federation, 2006). There was agreement that the criteria should include glucose intolerance, obesity, hypertension and dyslipidemia, however differences arose in certain areas. WHO, EGIR and AACE identify IR as a key part of MetS which is

diagnosed via oral glucose tolerance test (OGTT) or hyperinsulinemic-euglycemic clamp. Though this is the gold standard, it is a costly and labour intensive measure best used in a research laboratory under supervision. Another issue with WHO and NCEP ATPIII criteria is the lack of use of adiposity cut-offs with different ethnicities. WC cut-offs should account for the differences in fat distribution and risk of T2DM in different ethnic groups. The most recent definition from the IDF has taken this into account providing ethnic specific WC cut-offs. A recent comparison of the use of the WHO, ATPII, EGIR and IDF definitions in comparing the prevalence of MetS in Australia concluded that the IDF is the most useful in a clinical setting due to the ease of use of its criteria (Cameron, Magliano, Zimmet, Welborn, & Shaw, 2007). The WHO, EGIR, NCEP ATP III, AACE and IDF criteria are stated in Table 2.6.

Table 2.6 MetS criteria according to WHO, EGIR, NCEP: ATP III, AACE, and IDF.

Criteria	WHO (Alberti & Zimmet, 1998)	EGIR (Balkau & Charles, 1999)	NCEP: ATP III (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001)	AACE (Einhorn et al., 2003)	IDF (International Diabetes Federation, 2006)
Insulin resistance	IGT, IFG, T2DM or lowered insulin sensitivity; plus 2 additional components	IR or fasting hyperinsulinaemia >75th percentile	None, but three of the following five components	IGT or IFG, plus any other components	None
Adiposity	WHR >0.90 males, >0.85 females and/or BMI >30 kg/m^2	WC ≥94 cm males or ≥80 cm in females	WC ≥102 cm in males or ≥88 cm in females	BMI ≥25 kg/m^2	Europids males ≥94 cm, females ≥80 cm, ATSI, other ethnicity males >90cm, plus two additional components
Glucose	IGT, IFG or T2DM	IGT or IFG (FPG≥6.1 mmol/L), but non-diabetic	>110 mg/dL, includes diabetics	IGT and IFG, but not diabetic	FPG ≥100 mg/dL (5.6 mmol/L), or previously diagnosed T2DM
Lipids	TG ≥150 mg/dL (1.7 mmol/l) and/or HDL-C <35 mg/dL in men or <39 mg/dL in women	TG ≥150 mg/dL (1.7 mmol/l) and/or HDL-C <39 mg/dL in men or women	TG ≥150 mg/dL (1.7 mmol/l), HDL-C <40 mg/dL in males of <50 mg/dL in females	TG ≥150 mg/dL (1.7 mmol/l) and HDL-C <40 mg/dL in males of <50 mg/dL in females	TG ≥150 mg/dL (1.7 mmol/l) or treatment of TG, HDL-C <40 mg/dL (1.03 mmol/L) in males and <50 mg/dL (1.29 mmol/L) in females or treatment for HDL-C
Blood pressure (SBP/DBP)	≥140/90 mmHg	≥140/90 mmHg or treatment for hypertension	≥130/85 mmHg	≥130/85 mmHg	≥130/85 mmHg or treatment for hypertension

Criteria	WHO (Alberti & Zimmet, 1998)	EGIR (Balkau & Charles, 1999)	NCEP: ATP III (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001)	AACE (Einhorn et al., 2003)	IDF (International Diabetes Federation, 2006)
Other	Microalbuminuria: urinary excretion rate >20 mg/mon or albumin:creatinine ratio of 30 mg/g			Family history T2DM, polycystic ovary syndrome, sedentary lifestyle, older age, ethnic groups at higher risk of T2DM	

Footnotes: AACE, American Association of Clinical Endocrinologists; ATSI, Aboriginal and Torres Strait Islander; BMI, body mass index; DBP, diastolic blood pressure; EGIR, European Group for the Study of Insulin Resistance; FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol; IDF, International Diabetes Federation; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NCEP: ATP III, National Cholesterol Education Program's Adult Treatment Panel II; SBP, systolic blood pressure; TG, triglycerides; T2DM, type 2 diabetes mellitus; WC, waist circumference; WHR, waist:hip ratio; WHO, World Health Organization.

2.5.3 What causes MetS?

The exact cause of MetS has not been confirmed, however the most popular candidate is IR. Early on, MetS was referred to as the 'insulin resistant syndrome' indicating the major part that IR plays in MetS (Reaven, 1988a), however, it was soon realised that there may be other accompanying factors. IR has been found to influence the development of hyperglycemia, hypertension (Ferrannini et al., 1997; Reaven, 2003), hypertriglyceridemia (Ginsberg, 2002) and obesity (Nasser, 2009) through various mechanisms. However, some evidence had indicated that not all who have MetS, also have IR (Nasser, 2009). A factor analysis investigating the central condition of MetS found that it is a clustering of factors whereby central and visceral adiposity is a common factor (Anderson et al., 2001). When those with impaired glucose tolerance (IGT) were removed from the analysis, BP and obesity were the two common 'clustering' factors. A more recently identified factor in the MetS condition is inflammation. Adipose tissue is an active organ, and greater amounts of this tissue release pro-inflammatory factors resulting in a low grade inflammatory state (Festa et al., 2000). The state of chronic inflammation is common in obesity, and this may interfere with the anti-inflammatory effect of insulin increasing IR and stimulating atherogenesis. This may further perpetuate high BP and dyslipidaemia, indicating the complexity of each of the pathophysiological conditions present (Rana, Nieuwdorp, Jukema, & Kastelein, 2007). Although IR appears to be the primary causal factor of MetS, the link between IR and the other MetS components is complicated and may occur in both directions. IR may influence the development of hyperglycemia and dyslipidaemia, which may further aggravate IR (Nasser, 2009).

2.5.4 Why classify individuals as having MetS?

Those with MetS tend to be at higher risk of certain vascular diseases (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001) and death

than those without MetS (George, Alberti, Zimmet, & Shaw, 2005). More specifically, the classification of MetS was flagged as a useful tool for clinicians to identify those who may also be at risk of CVD and T2DM (Grundy, 2006). Those with MetS are predicted to have twice the risk of developing CVD and five times the risk of developing T2DM over the next decade (Alberti et al., 2009). MetS is also a primary risk factor for atherothrombotic events (Novo et al., 2013). This may allow for the implementation of therapeutic lifestyle changes, including diet and physical activity, and medications to ensure better health outcomes relative to CVD and T2DM (Reaven, 2004).

MetS classification is based upon the presence of three or more components, however each component is an independent pathophysiological condition. Both MetS and its individual components are predictive of future coronary heart disease, ischemic stroke, carotid artery disease and diabetes (Ballantyne et al., 2008). The risk of CVD and T2DM has been found to rise in parallel with the increase in the number of components present (Klein, Klein, & Lee, 2002). The risk of incidence CVD and T2DM after five years appears to increase from 2.5% and 1.1% respectively for those with one component to 14.9% and 17.9% for those with four or more components (Klein et al., 2002). Thus, focussing on MetS and its components are potentially equally as important in order to reduce the risk of associated diseases.

2.5.5 Prevalence of MetS

According to the IDF, approximately ~25% of the adults worldwide are classified as having MetS (International Diabetes Federation, 2006). However, the prevalence of MetS has also been broadly estimated as between <10% to 84%, dependent on a range of factors such as: age, sex, ethnicity, urban vs. rural environment, and the MetS criteria used (Desroches & Lamarche, 2004; Kolovou, Anagnostopoulou, Salpea, & Mikhailidis, 2007). Prevalence of MetS appears to increase with increasing age (Ford, Giles, & Dietz, 2002) . The rates of MetS appears to vary between gender with around 8% to 43% prevalence in males, and 7% to 56% prevalence in females (Cameron, Shaw, & Zimmet, 2004). Other factors that may affect the

prevalence of MetS include genetics, family history of diabetes, diet, physical activity, smoking status and education (Cameron et al., 2004). This large discrepancy in prevalence rates is mainly due to the lack of consensus on the use of a singular MetS definition. The agreement on one set of criteria would be highly beneficial and would provide: l) a tool for health professionals to identify those who are at greater risk of CVD and T2DM, and 2) epidemiologists and other health professionals in the area, comparable prevalence rates of MetS and the predictability of development of other chronic diseases (Desroches & Lamarche, 2004).

The comparability of prevalence of MetS is difficult due to the use of differing criteria. In Australia, a study of 11, 247 subjects found a variation in the prevalence rate of MetS when comparing four MetS definitions. The presence of MetS ranged from 22.1%, 21.7%, 30.7% and 13.4% (non-diabetic population only) when using ATPIII, WHO, IDF and EGIR definitions respectively (Cameron et al., 2007) . The prevalence of MetS also increased with age, with higher rates in males than in females in all definitions. Most recently the VHM employed the use of the joint consensus statement (Alberti et al., 2009) and estimated MetS prevalence to be 20.9% (Department of Health, 2012a). MetS appeared to increase with age, with higher proportion of MetS in males than in females, similar with previous findings (Department of Health, 2012a). Variations within definitions such as: abdominal obesity being the core component (NCEP: ATPIII) (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001) vs. impaired fasting glucose (IFG) and IR being core components (WHO, EGIR) (Alberti & Zimmet, 1998; Balkau & Charles, 1999), and WC cut-offs dependent on ethnicity (ATPIII) (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001) may affect prevalence rates. Regardless of the definition used, the rates of MetS remains high in Australia. Furthermore, the increased risk of future CVD and T2DM is also concerning.

2.5.6 Calcium, vitamin D and MetS and its individual components

Certain dietary patterns have been found to be potentially protective against MetS (Calton, James, Pannu, & Soares, 2014). However, recent evidence has indicated that a deficiency in certain nutrients, such as calcium and vitamin D, may contribute to the developments of MetS components. The potential mechanisms involved are complicated, however vitamin D status and calcium intake appear to be a key part. The majority of evidence looking at MetS components and its associations with vitamin D and calcium intake, tend be from the supplement perspective and very few also look at vitamin D concentration or dietary intake of calcium. Thus, the studies described in section 2.5.6.1, 2.5.6.2, 2.5.6.3, and 2.5.6.4 (Table 2.7) below are a combination of vitamin D status and dietary calcium intake studies, as well as supplementation studies and their association with MetS components.

2.5.6.1 Waist circumference/obesity[2]

The measurement of WC serves as a good proxy for central obesity and forms one of the criteria for MetS. In general terms a consistent observation from cross-sectional, longitudinal and clinical trials is that most measures of body fatness and its distribution (including WC) showed an inverse relationship to 25OHD (Caron-Jobin, 2011; Cheng et al., 2010; Gonzalez et al., 2015; Soares, Chan She Ping-Delfos, & Ghanbari, 2011a; Song & Sergeev, 2012). The direction of this association is most likely one where obesity is always associated with low 25OHD, while the reverse is less likely to be true (Vimaleswaran et al., 2014). Initially this association was explained by lower levels of physical activity and exposure to sunshine in obese individuals (Cheng et al., 2010). However, it appears that adipose tissue may 'sequester' the fat soluble vitamin resulting in a reduced bioavailability. Thus those with greater fat mass tend to have a lower vitamin D status.

[2] Sections 2.5.6.1; 2.5.6.2; 2.5.6.3,2.5.6.4; Table 2.7 of Chapter 2 have taken from an unpublished article: Pannu, P. K., Soares, M. J., Pathak, K., & Calton, E. K. The influence of calcium and vitamin D on components of the metabolic syndrome. Reproduced with permission from the authors (Appendix K).

The evidence to date from SR and MA have been highlighted in Table 2.7. Only one SR explored if there was any association between calcium intake and body fat, and showed a small significant effect on body fat during weight loss (Onakpoya, Perry, Zhang, & Ernst, 2011). However, the authors point out that the overall positive outcome was obtained on a small number of trials. Moreover, randomisation procedures were incompletely described as were allocation concealment, resulting in lower quality scores for these studies (Onakpoya et al., 2011). In addition the SR on vitamin D alone or in combination with calcium also did not favour these nutrients (Chandler et al., 2015; Manousopoulou, Al-Daghri, Garbis, & Chrousos, 2015; Pathak, Soares, Calton, Zhao, & Hallett, 2014; Soares et al., 2011a; Song & Sergeev, 2012) (Table 2.7). While the review of Pathak et al. (2014), selected studies on vitamin D supplementation in the absence of weight loss, the others included several trials with and without weight loss. Only one review investigated the association between vitamin D status, rather than supplementation, calcium intake (diet and supplements) and obesity outcomes. The authors found an inverse association between vitamin D status, calcium intake and body weight and fat, though the evidence was not definitive (Song & Sergeev, 2012). In conclusion, there does not appear to be consistent evidence to date that would support an additional effect of calcium and vitamin D on body weight status.

Some comment is appropriate here. Many of the older trials included in SR to date were initially designed with bone endpoints in mind and subsequently analysed for their potential effects on body composition. There is also is a great variation in body composition techniques and in their precision across weight loss studies (Erselcan, Candan, Saruhan, & Ayca, 2000) and similar issues arise with methods for measurement of 25OHD (Binkley et al., 2004; Black et al., 2014). Moreover, many SR have included studies with and without weight loss. The latter would blunt the likelihood of observing an effect, since vitamin D in the absence of caloric restriction did not influence weight or its composition (Pathak et al., 2014). It has been opined that there is a need to define a minimum threshold of 25OHD above which extra-skeletal effects are seen (Boucher, 2011; Dawson-Hughes et al., 2005; Vieth, 2006; Vieth et

al., 2007). While it is plausible that one cut-off may not suit all chronic diseases, two quality RCTs make the point quite well. Improvements in IR (von Hurst, Stonehouse, & Coad, 2010) and in weight loss parameters (Mason et al., 2014) were only significantly seen in those individuals who achieved a concentration of ~80 nmol/L. Not all studies in these reviews were aimed at a specific 25OHD concentration. Furthermore, it is now clear that at ~73 nmol/L, there is complete suppression of PTH (Durazo-Arvizu et al., 2010). This could be another key factor since higher PTH per se increases the risk of MetS (Ahlström et al., 2009; Huang, Shapses, & Wang, 2013; Soares et al., 2011a). Consequently, for an SR/MA to detect a weighted mean difference in favor of the treatment is difficult, especially if individual studies in SR were not consistent in their original endpoints, did not use comparable methodology or were not directed towards a target vitamin D level that ensured complete suppression of PTH.

2.5.6.2 Glucose[2]

Vitamin D and calcium play key roles in modulating glucose homeostasis. The nVDR is expressed in a range of tissues (Bouillon, Bischoff-Ferrari, & Willett, 2008), including the pancreatic β cells (Walters, 1992; Zeitz et al., 2003). The presence of the nVDR in the pancreatic tissue reflects an active and important role of vitamin D in insulin secretion. Insulin secretion is mediated by an intracellular increase in calcium concentrations and vitamin D may modulate this process (Feldman, 1999; Holick, 2003; Sergeev & Rhoten, 1995). Consequently a number of animal and human studies have indicated the possible involvement of calcium and vitamin D in glucose homeostasis and insulin action (Cavalier, Delanaye, Souberbielle, & Radermecker, 2011; Kayaniyil et al., 2010; Kositsawat, Freeman, Gerber, & Geraci, 2010; Maxwell & Wood, 2011; Pittas et al., 2010a). In partial support are observations that individuals with lower 25OHD tend to have impaired glucose control (Choi et al., 2011; Gagnon et al., 2012; Kayaniyil et al., 2011; Thomas et al., 2012; Vitezova et al., 2015; Yin et al., 2012; Zoppini et al., 2013) and that vitamin D and calcium supplementation may improve insulin sensitivity (Choi et al., 2011; Gagnon et al., 2012; Kayaniyil et al., 2011; Thomas et

al., 2012; Vitezova et al., 2015; Yin et al., 2012; Zoppini et al., 2013), beta cell function and glucose homeostasis (Harinarayan et al., 2014; Mitri, Dawson-Hughes, Hu, & Pittas, 2011a).

A number of cross-sectional studies have highlighted lower 25OHD levels in those with impaired glucose control (Choi et al., 2011; Kayaniyil et al., 2011; Vitezova et al., 2015; Zoppini et al., 2013). A large cross-sectional study found an inverse association between 25OHD and HbA1c levels in those aged 35-74 years, with no association in the 18-34 years and ≥75 years age group (Kositsawat et al., 2010). Some studies have found that vitamin D and calcium supplementation may improve beta cell function and in turn improve glucose homeostasis (Mitri et al., 2011a). Three cross-sectional and two longitudinal studies suggest a correlation between vitamin D status and FPG, HbA1c and IR, with two randomised controlled trials (RCTs) showing differing results of the impact of vitamin D and/or calcium intake and the risk of T2DM. The association between 25OHD, vitamin D and calcium intake and risk of higher FPG, HbA1c and IR indicated that there is a trend of lower 25OHD levels and higher FPG, HbA1c and IR. Majority of the aforementioned studies were cross-sectional in nature which means causality can not be ascertained. Study results may vary due to a number of confounding factors including: sun exposure, differences in the population (age, sex, ethnicity, geographical location), and variances in etiopathology and diabetic outcomes (newly diagnosed diabetics vs. diagnosed diabetics, FPG vs. HbA1c), measurement of 25OHD (predicted 25OHD score vs. serum 25OHD measurement) and dietary recall methods.

There have been a reasonable number of SR and MA in this area (Table 2.7). Overall three (Nigil Haroon, Anton, John, & Mittal, 2015; Poolsup, Suksomboon, & Plordplong, 2016; Zuk, Fitzpatrick, & Rosella, 2016) out of nine SR and MA indicated a beneficial effect of vitamin D supplementation on reducing HbA1c and FPG. The most recent SR and MA of RCTs in overweight and obese adults indicated positive effects in most of the 11 studies of vitamin D supplementation that measured FPG, HbA1c and IR (HOMA-IR and/or QUICKI) (Zuk et al., 2016). However, in two vitamin D and calcium supplementation studies found no effect on FPG and HbA1c compared to control. However on sub-group analysis of those with

impaired fasting glucose only, vitamin D and or calcium supplementation appeared to improve HbA1c (Zuk et al., 2016). A second MA of ten RCTs found that vitamin D supplementation significantly reduced HbA1c (-1 mmol/L) and FPG (-0.10 mmol/L) levels (Poolsup et al., 2016). A third SR of RCTs and longitudinal studies found than ten studies indicated an improvement in HbA1c after vitamin D supplementation in studies with a duration of ≤ 3 months (Nigil Haroon et al., 2015). In contract, the six remaining SR and MA of RCTs found no effect of vitamin D supplementation on HbA1c, FPG or IR (Elamin et al., 2011; George, Pearson, & Witham, 2012; Jamka et al., 2015; Manousopoulou et al., 2015; Pittas et al., 2010a; Seida et al., 2014).

A MA is a useful statistical approach to assess the cumulative findings of studies, with the use of a suitable model. Having said that, the ability to control for important confounders is not always possible due to the limited amount of information collected and reported in studies. A number of common factors may explain the lack of effect of vitamin D supplementation on glucose parameters in the MA of these RCTs. The sub-optimal and/or variable dosing (up to 12000 IU/d) (Jamka et al., 2015) of the vitamin may favour inconsistent results (Elamin et al., 2011; George et al., 2012; Jamka et al., 2015; Seida et al., 2014). Many studies were using <2000 IU/d, and some may argue the need for higher doses such as 5000 IU/d to increase 25OHD status. Results will also vary due to differences in the duration of the intervention (6 weeks to 3 years). The appropriate amount of time required to assess any effects that supplementation may have on glucose parameters has not been confirmed (George et al., 2012; Jamka et al., 2015; Seida et al., 2014). However, for a slow progressing states such as pre-diabetes, this may require longer term studies to evaluate the benefits (Seida et al., 2014), where most studies were of shorter duration. Lastly, the sample sizes of these RCTs tended to be small, which does not always provide sufficient statistical power to see an effect (George et al., 2012; Seida et al., 2014). A large body of cross-sectional evidence indicates a correlation between the nutrients and measures of T2DM risk, however a casual association needs to be further tested by good quality RCTs.

2.5.6.3 Lipids[2]

The classification of those with MetS involved the identification of those with raised TGs and low density lipoprotein-cholesterol (LDL-C) or lower HDL-C. Calcium may play a role in reducing risk of lipid abnormalities through two functions. Firstly, absorbed calcium strongly promotes fat oxidation (Soares et al., 2012) such that every 800 mg/d is predicted to increase fat oxidation by 11% (Gonzalez, Rumbold, & Stevenson, 2012). Secondly, dietary calcium that is unabsorbed from the gastrointestinal (GI) tract binds to luminal fatty acids to form calcium-fatty acid soaps. Hence calcium increases faecal fat loss to the tune of 5 g/d for every 1200 mg/d (Christensen et al., 2009). These potential pathways may explain the early observations that increases in calcium intake with or without vitamin D may result in lower circulating TGs and other lipid fractions (Jacqmain, Doucet, Després, Bouchard, & Tremblay, 2003; Major, Alarie, Dore, Phouttama, & Tremblay, 2007; Reid et al., 2002).

Evidence from cross sectional studies clearly indicate that higher calcium and/or vitamin D are associated with a more favourable lipid profile. Amongst all lipid fractions, the most consistent effect was found with TGs (Jorde & Grimnes, 2011; Zittermann, Gummert, & Börgermann, 2011). A recent large lipid study of >20,000 patients from one database concluded that vitamin D was strongly associated with decreases in all lipid fractions (Lupton et al., 2016). These results contrast with two SR of RCTs on vitamin D supplementation (Table 2.7) (Elamin et al., 2011; Wang, Xia, Yang, & Peng, 2012). Wang et al. (2012) which found an additional effect on vitamin D over the effects of weight loss alone and however found no effect on TG, HDL-C and total cholesterol. Surprisingly they note an increase in LDL-C following the vitamin D arm. Few studies have looked at vitamin D status and lipid outcomes. Two SR found an association between lower vitamin D status and higher TG, lower HDL-C (Manousopoulou et al., 2015; Zittermann et al., 2011).

A lack of intervention studies as well as varying results means it is difficult to draw a conclusion. The variance in vitamin D given, such as the amount and formulation (vitamin D3 vs. vitamin D2 vs. alpha-calcidol), make it difficult to compare study results (Wang et al.,

2012). One SR indicated that the shorter duration studies tended to have a more noticeable effect on LDL-C than longer duration potentially due to poor compliance as time progresses (Wang et al., 2012). Conversely, longer term studies appeared to indicate more significant changes in HDL-C levels than shorter duration studies. Certain limitations from studies included a lack of control for confounders (eg. no adjustment for gender when analysing male and female data) (Manousopoulou et al., 2015), small sample sizes, limited variance in studies in terms of cultural groups and age groups (mainly elderly) (Wang et al., 2012; Zittermann et al., 2011), and differences in the geographical latitudes of study areas (Wang et al., 2012). The effect of vitamin D on lipid profiles appears to be divergent thus more evidence is required to determine the optimal vitamin D level for favourable lipid outcomes.

2.5.6.4 Blood pressure[2]

The benefit of higher dietary calcium intake and lower BP has been noted previously (McCarron & Reusser, 1999). Calcium plays an essential role in the contraction and relaxation of normal muscle. Calcium (Ca2+) -activated Cl(-) channels (CaCCs) are essential for the functioning of blood vessels, kidney and the heart (Matchkov, Boedtkjer, & Aalkjaer, 2015). Consequently, adequate amounts of dietary calcium are required for the maintenance of appropriate BP levels through regulation of vascular resistance. Potential pathways that may explain this relationship may be that higher calcium intake may suppress PTH and $1,25(OH)_2D$, resulting in adipocyte metabolism by inhibiting lipogenesis and stimulating lipolysis (Zemel, Thompson, Milstead, Morris, & Campbell, 2004). Vitamin D assists in regulating cardiovascular outcomes via activation of renin angiotensin system (RAS), improve vasodilation, and inhibit proliferation of vascular cell smooth muscle (Li et al., 2004; Nemerovski et al., 2009). Low vitamin D levels are associated with raised BP via the activation of the RAS. The activation of the RAS and production of angiotensin is a vitamin D dependent mechanism, and is responsible for increasing vascular tone and arterial stiffness. These two factors are precursors in the development of hypertension. Consequently, low serum vitamin

D concentration may affect the activation of RAS resulting in raised BP (Al Mheid, Patel, Tangpricha, & Quyyumi, 2013).

A few observational studies have found an inverse association between dietary calcium and BP levels or a decreased risk of developing hypertension (Schutte et al., 2010; Yao et al., 2013). In a two year study, it was observed that those with low baseline calcium intakes (785 mg/d) and calcium supplementation (1200 mg/d) showed a decrease of -4.2 mmHg in SBP and -3.3 mmHg in DBP vs. placebo. There was no effect on BP in those with baseline calcium intakes of >785 mg/d plus supplementation (Reid et al., 2010). An RCT on postmenopausal women, found no effect of 1000 mg/d of calcium on blood pressure after three months vs. placebo as compared to the control. Furthermore, the reduction in BP was smaller in the calcium supplementation group than the control group (Bristow, Gamble, Stewart, Horne, & Reid, 2015).

Two SR and MA explored the association between vitamin D status and the risk of hypertension (Table 2.7) (Burgaz, Orsini, Larsson, & Wolk, 2011; Kunutsor, Apekey, & Steur, 2013), and found that those in higher tertiles of 25OHD had a 27% (Burgaz et al., 2011) to 30% lower risk of developing hypertension (Kunutsor et al., 2013). More recently, a large MA using a mendelian randomisation approach, reported that every 10% increase in 25OHD concentration was associated with a decrease of -0.29 mmHg in DBP and decrease of -0.37 mmHg in SBP (Vimaleswaran et al., 2014). Conversely one of the most comprehensive reviews of vitamin D supplementation found no effect in lowering BP (Beveridge et al., 2015). In addition, a SR and MA of non-diabetic obese subjects showed a significant increase in SBP after vitamin D supplementation (Manousopoulou et al., 2015). However, the duration (from six weeks to four years) and dosage (1000 IU/d to 120 000 IU fortnightly) were highly varied. In one study, participants were advised to increase sunlight exposure and dietary vitamin D intake. To conclude, the debate continues around whether or not vitamin D supplementation supports the improvement in hypertension. All these studies indicate the need of more intense clinical trials to define, dosage, duration and form of vitamin D required to improve BP.

Table 2.7 Studies on vitamin D and calcium intake and MetS components.

Authors and year of publication	Study design and duration	Intervention	Outcome
Fat mass/waist circumference			
Soares et al. (2011a)	Review of RCTs, 2000 – 2011 (weight loss).	Vitamin D ± Calcium vs. placebo	No effect on fat loss.
Onakpoya et al. (2011)	SR and MA of RCTS, inception – 2010 (weight loss).	Calcium vs. placebo	↑Weight loss and ↑fat loss.
Song and Sergeev (2012)	Review of RCTs, no date stated (with/without caloric restriction).	Vitamin D ± Calcium vs. placebo	No effect on weight and FM.
Pathak et al. (2014)	SR and MA of RCTs, 1995 – 2013 (without caloric restriction).	Vitamin D alone (various doses)	No effect on FM, %FM, and FFM.
Chandler et al. (2015)	SR and MA of RCTs, 1996 – 2014 (weight loss).	Vitamin D ± calcium vs. calcium or placebo	No effect on weight and FM.
Manousopoulou et al. (2015)	SR and MA of intervention studies, inception – 2014.	Vitamin D vs. with/without placebo	No effect on weight.
Glucose/HbA1c			
Pittas et al. (2010a)	SR of longitudinal observational cohort and RCTs, 1984 – 2009.	Vitamin D ± calcium	No effect on FPG.
Elamin et al. (2011)	MA and SR of RCTs, inception – 2010.	Vitamin D	No effect on glucose.
George et al. (2012)	MA and SR of RCTs, 1984 – 2011.	Vitamin D	No effect on HbA1c or FPG.
Seida et al. (2014)	MA and SR of RCTs, inception – 2013.	Vitamin D ± calcium	No effect on HbA1c or FPG.
Poolsup et al. (2016)	SR and MA on RCTs, inception – 2014.	Vitamin D –treatment	↓FPG and ↓HbA1c. No effect on IR.

Authors and year of publication	Study design and duration	Intervention	Outcome
Nigil Haroon et al. (2015)	SR of RCTs and longitudinal studies, inception – 2014.	Vitamin D	↓HbA1c in studies of ≤3 months but not in studies >3 months.
Jamka et al. (2015)	SR and MA of RCTs, 2006 – 2014.	Vitamin D	No effect on insulin or glucose.
Manousopoulou et al. (2015)	SR and MA of intervention studies, inception – 2014.	Vitamin D vs. with/without placebo	No effect on IR.
Blood pressure			
Pittas et al. (2010a)	SR of longitudinal observational cohort and RCTs, 1984 – 2009.	Vitamin D ± calcium	No effect on SBP or DBP.
Wu, Ho, and Zhong (2010)	SR and MA of RCTs, 1947 – 2009.	Vitamin D ± calcium vs. calcium or placebo	↓SBP. No effect on DBP.
Burgaz et al. (2011)	MA of prospective and cross-sectional studies inception-2010.	Vitamin D	↓ hypertension in higher category of 25OHD.
Elamin et al. (2011)	SR and MA of RCTs, inception – 2010.	Vitamin D	No effect on SBP or DBP.
Uusi-Rasi, Karkkainen, and Lamberg-Allardt (2013)	SR of MA, RCTs and cohort studies, 2000 – 2011.	Calcium	↓SBP in pregnancy and in hypertension.
Kunutsor et al. (2013)	SR and MA of prospective studies, 1950 – 2012.	Vitamin D	Every 10 ng/ml increment 25OHD reduce risk of hypertension by 12%.
Manousopoulou et al. (2015)	SR and MA of intervention studies, inception – 2014.	Vitamin D vs. with/without placebo	↑SBP. No effect on DBP.
Beveridge et al. (2015)	SR and MA on RCTs, 1966 – 2014.	Vitamin D vs. placebo	No effect on SBP and DBP.
Golzarand, Shab-Bidar, Koochakpoor, Speakman, and Djafarian (2016)	SR and MA on RCTS, inception – 2015.	Vitamin D	No effect on SBP and DBP.
Lipids			

Authors and year of publication	Study design and duration	Intervention	Outcome
Elamin et al. (2011)	MA and SR of RCTs, inception – 2010.	Vitamin D	No effect on TG, HDL-C, LDL-C.
Zittermann et al. (2011)	SR of various studies, inception – 2010.	Vitamin D	Inverse relation with TG, especially with high baseline TG.
Wang et al. (2012)	MA of RCTs, inception – 2011.	Vitamin D ± calcium – treatment Calcium or placebo-control	No effect on TC, HDL-C, and TG. Significant ↑LDL-C.
Manousopoulou et al. (2015)	SR and MA of intervention studies, inception – 2014.	Vitamin D vs. with/without placebo	↑ LDL-C, but ↓TG. No effect on HDL-C.

Footnotes: DBP, diastolic blood pressure; FPG, fasting plasma glucose; FM, fat mass; %FM, percentage fat mass; FFM, fat free mass; HbA1c, glycated haemoglobin; HDL-C, high density lipoprotein cholesterol; IR, insulin resistance; LDL-C, low density lipoprotein cholesterol; MA, meta-analysis; RCT, randomised controlled trials; SR, systematic review; SBP, systolic blood pressure; TG; triglycerides; TC, total cholesterol; 25OHD, 25-hydroxyvitamin D.

2.6 The gap

Majority of studies have investigated vitamin D and its association with MetS, however few have included dietary calcium intake. In addition, limited studies have explored MetS according to the individual components, though this is important as they are considered to be independent pathophysiological conditions. The vitamin D-calcium relationship is tightly intertwined, and both nutrients are involved in various mechanisms underscoring MetS and its components. Thus it is only logical to explore if there is any association between serum vitamin D concentration, dietary calcium intake and the combination of both on MetS and its components. In the current climate of high levels of vitamin D deficiency, inadequate calcium intakes and increasing rates of MetS, this area requires further investigation. There is limited Australian evidence of the relationship between vitamin D, calcium and Mets. To the best of our knowledge, no studies in Australia have explored the combined effect of serum vitamin D

concentration and dietary calcium intake on MetS and its components. The combination of the nutrients may be more beneficial on Mets and its components than the independent effect of each.

2.7 Type 2 diabetes

2.7.1 Current definition of T2DM

Diabetes is a common form of vascular disease, and can be defined as a chronic state of hyperglycaemia (American Diabetes Association, 2010; Paneni, Beckman, Creager, & Cosentino, 2013). T2DM may remain undiagnosed until an abnormal blood or urine glucose test is detected (Paneni et al., 2013), and indicates the bodies decreasing ability to metabolise glucose (Lupi & Del Prato, 2008). It has now been established that T2DM results due to two pathogenetic defects: beta cell dysfunction and IR (Alejandro, Gregg, Blandino-Rosano, Cras-Meneur, & Bernal-Mizrachi, 2015; Lupi & Del Prato, 2008). Genetic influences as well as modifiable risk factors such as diet, physical activity, and more so obesity may also be early preceding factors (Calpis & Frangopoulos, 2012).

According to the ADA, T2DM is diagnosed as per the cut-offs in Table 2.8. In the first category those who are at 'increased risk of T2DM' are those who do not meet the criteria for T2DM however have higher than normal glucose levels. Those with FPG of 5.6-6.9 nmol/L are considered as having IFG, or those with 2h-plasma glucose (PG) during OGTT of 7.8-11.0 nmol/L are considered as having IGT. Thus, those with IFG and/or IGT are classified as having 'pre-diabetes' and are 'at increased risk of T2DM' in the future. HbA1c is an indicator of glucose levels over a two to three month period. Those with HbA1c of 5.7-6.4% are also at increased risk of future T2DM. All those at increased risk of T2DM are also at risk of CVD due to inter-related pathophysiology (American Diabetes Association, 2010). A 'diagnosis of T2DM' is based upon having FPG ≥7.0 mmol/L, HbA1c ≥6.5%, a 2hr-PG during OGTT of ≥11.1 mmol/L or having symptoms of hyperglycemia. However, HbA1c is a better indicator of those at risk of T2DM as it provides a longer-term trend.

Table 2.8 American Diabetes Association criteria for those at increased risk of T2DM and the diagnosis of T2DM.

	Increased risk of T2DM	Diagnosis of T2DM
HbA1c	5.7-6.4%	≥6.5%
FPG	5.6-6.9 nmol/L	≥7.0 mmol/L
2h-PG during OGTT	7.8-11.0 mmol/L	≥11.1 mmol/L
		Symptoms of hyperglycemia or hyperglycemic crisis, random plasma glucose ≥11.1 nmol/L

Footnotes: FPG, fasting plasma glucose; h, hours; HbA1c, glycated haemoglobin; OGTT, oral glucose tolerance test; PG, plasma glucose; T2DM, type 2 diabetes mellitus.

2.7.1.1 Obesity and T2DM

Obesity is a key player in the manifestation of T2DM, where approximately 60-90% of diabetes occurrences are as a result of high body weight (Anderson, Kendall, & Jenkins, 2003). Obesity drives the pathogenesis of T2DM, which in turn influences beta cell dysfunction and IR (Kasuga, 2006). The issue of which comes first is still not clear. However, the key association between obesity and T2DM is that obesity encourages progressive IR, which is followed by a decline in beta cell function, thus resulting in hyperglycaemia (Kasuga, 2006). It should be noted that, obesity-related IR is a complex association consisting of various endocrine, inflammatory, neural and cell intrinsic pathways that are dysregulated in obesity (Qatanani & Lazar, 2007).

2.7.1.2 Beta cell dysfunction

The pancreas is a glandular organ in the upper abdomen, but really it serves as two glands in one: a digestive exocrine gland and a hormone-producing endocrine gland. The pancreas contains beta cells, which produce and secrete insulin, a key hormone in maintaining strict controls over plasma glucose concentration (Ferrannini & Mari, 2014; Gastaldelli, 2011). Though a small organ, the pancreas can potentially store 200-250 units of insulin, which equates to a ten day supply for a healthy individual (Rahier, Guiot, Goebbels, Sempoux, &

Henquin, 2008). In healthy subjects, sufficient insulin is released from beta cells after a meal is consumed, in response to variations in glucose concentration (Gastaldelli, 2011). Beta cells are adaptive, and insulin is secreted according to post-meal variation (Ferrannini & Mari, 2014), however over working of cells may lead to a decrease in beta cell function (Lebovitz, 2000). In those with persistent hyperglycemia a decrease in beta cell mass coupled with beta cell 'exhaustion' due to constant secretion of insulin, results in beta cell dysfunction (LeRoith, 2002).

Initially in the progression of T2DM, beta cell dysfunction involves: the inadequate response of beta cells to glucose, and then a progressive reduction in beta cell mass via increased apoptosis and decreased regeneration occurs (Gastaldelli, 2011; LeRoith, 2002). The underlying mechanisms of beta cell dysfunction has not been confirmed, but several factors may be responsible. A genetic angle indicates that increased apoptosis and reduced regeneration of beta cells may be due to genetic programming (Lebovitz, 2000). Secondly, during the early stages of IR, the continuous exertion on insulin secretion may increase beta cell death (Lebovitz, 2000; Maedler et al., 2001). Lastly, chronic hyperglycaemia may encourage beta cell death as a result of glucose toxicity pathways (Lebovitz, 2000; LeRoith, 2002). Consequently, T2DM will only develop in the presence of beta cell abnormalities. Beta cell dysfunction does not act independently, and the secondary factor in the development of T2DM is IR.

2.7.1.3 Insulin resistance

Insulin is a specialised hormone and is secreted by the pancreas according to size, composition and appearance rate of meals (Ferrannini & Mari, 2014). On consumption of a large meal, insulin secretion can fluctuate within a few minutes, or over a longer period of time, such as in progressive accumulation of weight (Ferrannini & Mari, 2014). In the latter situation, higher concentrations of insulin are required to dispose of glucose (Goldstein, 2002). IR arises as body tissues are no longer sensitive to insulin action. In those at risk of T2DM, IR

demands higher amounts of insulin from beta cells (Gastaldelli, 2011). Continuous consumption of high calorie foods spikes blood glucose levels and requires continual insulin secretion to maintain normal glucose levels. Over time, beta cells become 'exhausted' as insulin production is not adequate to normalise glucose levels (Staimez et al., 2013). Thus IR can be described as a lack of insulin to maintain plasma blood glucose levels or, decreased insulin sensitivity by tissues (Gastaldelli, 2011). IR and beta cell dysfunction are the key players in the development of T2DM, where obesity is a definite pre-cursor.

2.7.2 Prevalence of T2DM[1]

According to the IDF (International Diabetes Federation, 2006), 8.3% of adults or 382 million people worldwide have diabetes. This figure is projected to increase by 55% to over 592 million people by 2035. Of most concern are the 175 million individuals who are currently undiagnosed and progressing toward potential diabetes-related complications. Diabetes tends to be more prevalent in lower to middle income countries, with four out of every five people diagnosed with diabetes. In 2013, diabetes was estimated to cause 5.1 million deaths which equates to a mortality every six seconds (International Diabetes Federation, 2006). Certain ethnic groups, especially those from India and China, are at higher risk of T2DM than others (Chiu, Cohan, Lee, & Chuang, 2000; Gujral, Narayan, Kahn, & Kanaya, 2014; Misra et al., 2010; Shaw, Sicree, & Zimmet, 2010). There are a few key reasons for this increased risk including specific genes that may increase the risk of T2DM through influencing beta cell dysfunction and IR (Ferrannini, 1998; Flores, 2005), and a higher tendency of central adiposity. Obesity is a primary driver of T2DM pandemic (Figure 1.1), since approximately 60–90% of diabetes incidence is the result of higher body weight (Anderson et al., 2003).

2.7.3 Association between dietary calcium intake and T2DM[1]

Prospective studies have found varying results between calcium intakes and risk of T2DM (Colditz et al., 1992; Pittas et al., 2006; van Dam, Hu, Rosenberg, Krishnan, & Palmer, 2006) (Table 2.6). A large prospective cohort study in 41,186 subjects found that higher calcium

intake was not associated with risk of T2DM. In contrast, those who consumed calcium supplements had a decreased risk of T2DM compared to non- supplement users. However, within supplement users there was no association between the amounts or duration of calcium supplement and a lower those who consumed <600 mg/d. When looking at daily calcium intake via supplements only, there was an 18% lower risk of diabetes in those who consumed ≥500 mg vs. those who consumed ≤250 mg (Pittas et al., 2006). A MA of these two prospective studies (Pittas et al., 2006; van Dam et al., 2006) found an 18% decrease in the risk for incident T2DM in the highest (661–1200 mg) vs. the lowest calcium (219–600 mg) intake groups (Pittas et al., 2007). Though evidence on calcium intake and risk of diabetes is conflicting, there does appear to be a potential link between the two. Optimal intakes of calcium in order to reduce risk of T2DM have not been confirmed, however a MA indicates calcium intakes of more than 600 mg/d are desirable, with intakes over 1200 mg/d being ideal (Pittas et al., 2007). Inconclusive evidence around calcium intake via supplements and risk of T2DM requires high quality RCTs to indicate a casual link.

2.7.4 Association between vitamin D status and T2DM[1]

Vitamin D has numerous endocrine and autocrine roles however one of the key roles of the vitamin is to maintain phosphate and calcium homeostasis via enhancing absorption in the gut (Cavalier et al., 2011). The importance of vitamin D is indicated by the presence of the nVDR in numerous tissues, and the vitamin D system regulating around 3% of the human genome (Bouillon et al., 2008). The expression of the nVDR in pancreatic beta cells supports its physiological role of vitamin D in beta cell function (Bouillon et al., 2008; Jorde et al., 2012; Takiishi, Gysemans, Bouillon, & Mathieu, 2010).

A number of prospective (Forouhi, Luan, Cooper, Boucher, & Wareham, 2008; Husemoen et al., 2012), and cross-sectional studies (Chiu, Chu, Go, & Saad, 2004; Chonchol & Scragg, 2007; Del Gobbo, Song, Dannenbaum, Dewailly, & Egeland, 2011; Kayaniyil et al., 2011; Liu et al., 2009; Lu et al., 2009; Scragg, Sowers, & Bell, 2004) have indicated an

inverse association between 25OHD levels and risk of T2DM. A recent MA of 15 prospective studies indicated a significant inverse association between 25OHD levels and risk of T2DM, when comparing the highest 25OHD category vs. the lowest category (Song et al., 2013). In addition, every 10 nmol/L increment in 25OHD reduced the risk of T2DM by 4%. There appeared to be a significantly lower risk of T2DM when 25OHD status reached 50 nmol/L. The Institute of Medicine (2011) recommends a 25OHD status of 50–75 nmol/L for good bone health however sufficient 25OHD levels for T2DM prevention have not been confirmed. One clinical study indicated a positive association between 25OHD levels and insulin sensitivity, and a negative association with plasma glucose concentration (Chiu et al., 2004). A negative effect of low 25OHD levels on beta cell function was indicated by the relationship between 25OHD and plasma glucose concentration. This suggests that those with lower 25OHD levels affected beta cell function by inhibiting the usual compensatory insulin response that would control glucose concentrations. Thus those with lower 25OHD levels may have inhibited beta cell function thus higher plasma glucose levels. Similarly, a cross-sectional study found that those in the highest tertile of 25OHD (64 nmol/L) had significantly decreased FPG, fasting plasma insulin, and IR as compared to those in the lowest tertile of 25OHD (30 nmol/L) (Liu et al., 2009). Another study indicated that there was a fourfold increase in the risk of having T2DM in those in the lowest quartile (≤43.9 nmol/L) of vitamin D compared to those in the highest quartile (≥80 nmol/L) (Scragg et al., 2004). This indicates the critical role that vitamin D has by affecting insulin sensitivity, beta cell function, or both, thus low vitamin D levels are a potential risk factor for developing T2DM. However, the impact of other physiological mechanisms may also affect the risk of T2DM.

With vitamin D deficiency being a potential risk factor in developing T2DM, vitamin D supplementation would be a plausible solution. Unfortunately, a recent MA indicated that vitamin D supplementation trials are inconsistent in showing any changes in diabetes risk or glucose intolerance (Table 2.9) (George et al., 2012; Mitri et al., 2011a). One MA indicated that there was no significant effect of supplementation on FPG, HbA1c, and IR. When looking

at studies with subjects who already had IGT or T2DM, a small but significant effect was found with supplementation and FPG, and IR. Thus the author states that the results are of debatable clinical significance (George et al., 2012). A second MA looked at those with glucose intolerance and those with T2DM separately. Results indicated no effect on glycemic outcomes in those with T2DM however there was an improvement in IR in those with glucose intolerance (Mitri et al., 2011a). A third MA (Seida et al., 2014) also found no significant effect of vitamin D supplementation on glucose homeostasis or diabetes prevention. A number of limitations were identified in these reviews including a limited number of trials (George et al., 2012; Mitri et al., 2011a), small number of patients (George et al., 2012; Mitri et al., 2011a; Seida et al., 2014), variable study quality (George et al., 2012; Nigil Haroon et al., 2015; Seida et al., 2014), suboptimal dosing of vitamin D (George et al., 2012; Seida et al., 2014), type of vitamin D supplementation (oral vs. intra- muscular, ergocalciferol vs. calcitrol or cholecalciferol) (Nigil Haroon et al., 2015), and short treatment period and duration of follow-up (Nigil Haroon et al., 2015; Seida et al., 2014). In conclusion vitamin D deficiency may increase IR and reduce insulin secretion from beta cells. However, the effect of vitamin D supplementation on reducing the risk of T2DM is inconclusive.

Table 2.9 The effects of supplementing calcium and vitamin D on insulin resistance and type 2 diabetes: summary of systematic reviews and meta-analysis[1].

Authors and year of publication	Study design and duration	Intervention	Outcome
Vitamin D and calcium			
Pittas et al. (2007)	SR and MA of observational studies and clinical trials from 1994-2007.	Vitamin D ± calcium	No effect on T2DM.
Pittas et al. (2010a)	SR of various trials from 1984-2009.	Vitamin D ± calcium	No effect on FPG, HbA1c, T2DM incident.
Mitri et al. (2011a)	SR and MA on observational studies and RCTs from 1984-2010.	Vitamin D ± calcium	Vitamin D only. MA: Significant decrease in risk of developing T2DM in >500 IU/d vs. <200 IU/d (3 trials). Improved IR (2 trials). SR: RCTs no effect. Vitamin D ± calcium - no effect.
Vitamin D			
George et al. (2012)	SR and MA of RCTs from 1984-2011.	Vitamin D (various doses) vs. placebo	Small but significant decrease FPG. Small but significant improvement IR. No effect on HbA1c.
Seida et al. (2014)	SR and MA on RCTs from 1984-2013.	Vitamin D	No effect on IR, HbA1c, FPG, T2DM incident, beta cell dysfunction.

Nigil Haroon et al. (2015)	SR on RCTs and longitudinal studies from 2003-2014.	Vitamin D	Short term studies (<3 months): supplementation may have positive impact on glycemic control, IR, beta cell dysfunction. Long term (>3 months): no effect on HbA1c, beta cell function, IR.

Footnotes: d, day; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; IR, insulin resistance; MA, meta-analysis; RCT, randomised controlled trial; SR, systematic review; T2DM, type 2 diabetes mellitus.

2.8 The gap

The diagnosis of MetS, tends to increase the risk of certain chronic diseases including T2DM. In addition, lower vitamin D levels tend to increase the risk of developing T2DM. Only one study (Vitezova et al., 2015) accounted for the effect of all MetS component, when exploring the association between vitamin D status and T2DM. Thus, this area requires a stronger evidence base that confirms the effect of vitamin D status on T2DM, after controlling for MetS or its components.

Chapter 3 The determinants of vitamin D status of Australian adults aged 18-75 years

Objective addressed

Objective 2: To investigate the physical, demographic, and lifestyle determinants of vitamin D status in a population based sample of Australian adults aged 18-75 years.

3.1 Introduction

Vitamin D insufficiency is a serious disorder with around 1 billion people worldwide having sub-optimal levels (Holick, 2007). Vitamin D is required for optimal bone mineral density (Touvier et al., 2015), however more recently, its role in reducing the risk of hypertension, CVD, MetS, T2DM and multiple sclerosis have been proposed (Rosen, 2011; Vimaleswaran et al., 2014). Though the cut-offs for extra-skeletal health benefits have not been firmly established, with current indications that serum 25OHD levels ≥ 75 nmol/L for adults would be necessary (Bischoff-Ferrari et al., 2006; Heaney et al., 2009; Norman & Bouillon, 2010). In this regard a recent population based study (Daly et al., 2012) found that despite Australia being a sun-drenched country, 31% of the population had 25OHD levels <50 nmol/L while 73% had <75 nmol/L. Thus, vitamin D status could be critically low across all age groups in Australia; a matter of public health urgency.

There are a range of factors that influence 25OHD levels, including physical, demographic, and lifestyle determinants (Lips et al., 2014; Touvier et al., 2015; Tsiaras & Weinstock, 2011). Physical factors that may affect 25OHD levels include body weight and skin colour (Lips et al., 2014; Vimaleswaran et al., 2013). Accumulating evidence has found that BMI is inversely related to vitamin D status (Vimaleswaran et al., 2013); potentially a result of volumetric dilution of the fat soluble vitamin and its sequestration in adipose tissue (Drincic et al., 2012; Pannu, Zhao, & Soares, 2016b; Wortsman et al., 2000). Pigmentation in the skin acts as a sunscreen; with those with darker skin pigmentation having a lower 25OHD status (Clemens, Adams, Henderson, & Holick, 1982). Demographic factors such as age, gender, income, education, and residential location (i.e. residing in a metropolitan or rural area), can also affect 25OHD status in different ways. Those with a higher socio-economic status (SES) may have better 25OHD status due to an increased awareness of the benefits of sufficient 25OHD levels (Naugler, Zhang, Henne, Woods, & Hemmelgarn, 2013), as well as means to access health professionals for assessment of 25OHD status. A greater proportion of those residing in metropolitan areas tend to be vitamin D deficient, possibly due to more time

spent indoors, as compared with their rural counterparts (Bailey, Manning, & Peiris, 2012). Geographical factors such as UVB radiation, is key to generating the vitamin, as sun exposure is a major factor that determines vitamin D status. There is a seasonality to vitamin D status in Australia and this would be linked to the availability of sunny days that vary with season in different parts of the country (Bolland et al., 2008; Van Schoor et al., 2014).

Lifestyle factors such as physical activity, sitting time, smoking status, and dietary factors such as alcohol intake and dietary calcium consumption, have also been linked with vitamin D status. Physical activity has been found to be associated with higher vitamin D status (Freedman et al., 2013). However, it is not clear if it is due to sun exposure from outdoor activities, or if physical activity increases, $1,25(OH)_2D$ levels, which is the active form of vitamin D (Hibler et al., 2016; Zittermann et al., 2000), through the synthesis, absorption and metabolism of 25OHD (Scott et al., 2010). Conversely increased sitting time, whether at work or home, may decrease 25OHD due to limited sun exposure, though this factor has not been well explored (Daly et al., 2012; Hibler et al., 2016). 25OHD and calcium are tightly linked, whereby vitamin D status is dependent on and affected by any variations in calcium intake (Heaney, 2008).There is conflicting evidence surrounding alcohol consumption patterns and vitamin D. A recent review found that a similar number of authors reported a positive, negative and no association between the volume of alcohol consumed and 25OHD levels (Tardelli, Lago, Silveira, & Fidalgo, 2017). The link between alcohol intake and 25OHD is not clear, however some suggest that alcohol may affect absorption of 25OHD, or those who consume alcohol may have lower levels of sunlight exposure (Tardelli et al., 2017). Overall, factors such as low SES, physical inactivity, alcohol intake and smoking may congregate together. The reasoning behind this is complex, however lower educational attainment, limited economic means and an environment conducive to these unhealthy behaviours may explain some of the differences (Pampel, Krueger, & Denney, 2010). Therefore, our aim was to identify the physical, demographic, and lifestyle determinants of vitamin D status in a population representative sample of Australian adults.

3.2 Methods

3.2.1 Overview of VHM survey methods

Data collected by VHM survey, which was a state-wide population-representative survey of adults, aged 18-75 years, residing in Victoria, Australia, were used for this analysis. Participants were randomly selected from 50 metropolitan and rural Census Collection Districts (CDs), of Victoria. Non-institutionalised adults living in private dwellings were eligible for participation. Pregnant women, or people with intellectual disabilities or infirmity were not included in this survey. Data were collected over 12 months from May 2009 to April 2010. Data collection included three phases which were: a household interview, a biomedical examination, and a diet recall survey. The household interview included an interviewer-administered questionnaire to collect demographic and lifestyle variables. One eligible person from each household was randomly selected to participate in the survey. The biomedical examination included blood sampling and laboratory procedures, the physical examination included anthropometry, and the dietary data included three 24 hour dietary recalls. Further information on the biomedical examination, physical examination and dietary recalls is described below, and in the VHM report (Department of Health, 2012a) and VHM Food and Nutrition report (Department of Health, 2012b).

3.2.2 Sample[3]

We excluded participants with missing information on HbA1c data (n=31), those with HbA1c ≥6.7% as they were classified as having T2DM according to the American Diabetes Association (ADA) cut-offs (n=39) (American Diabetes Association, 2010), those with diagnosed T2DM (n=140), those with type one diabetes (T1DM) (n=9), and those on diabetic

[3] Sections 3.2.2; 3.2.3; 3.2.4; 3.2.5; 3.2.6; 3.2.7, 3.2.8 of Chapter 3 have been taken from a published article: Pannu, P. K., Zhao, Y., Soares, M. J. Piers, L. S., Ansari, Z. (2016). The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor Survey. Public Health Nutrition Research. 24, 1-12. (Appendix C). Reproduced with permission from the publisher (Appendix I) and authors (Appendix J).

medications (n=25). Thus, out of 3,653 participants collected in VHM survey, this analysis used a sample consisting of 3,409 adults, aged 18-75 years. Information on calcium and vitamin D supplements were not collected.

3.2.3 Assessment of vitamin D status[3]

Blood samples were collected via venepuncture, after an overnight fast of ten hours or more. The blood sample was immediately transported to an accredited central laboratory in Melbourne, Australia. The measurement of serum 25OHD concentration was based on DiaSorin Corporation Liaison® 25OHD total assay. The assay is an automated direct competitive chemiluminescent immunoassay that measures D_2 and D_3 to provide a total value for circulating vitamin D in nmol/L. The detection limit was 10 nmol/L. The All Laboratory Trimmed Mean (ALTM) was not computed by the laboratory, nor were results compared to a 'Target Value' (TV) assigned by the NIST Reference Measurement Procedure.

3.2.4 Assessment of dietary calcium intake[3]

Dietary intake data was obtained by multiple-pass 24 hour diet recall using computer assisted telephone interviews (CATI). The first diet recall interview was conducted within five to seven days of the participants attending the biomedical examination. Two subsequent diet recall interviews were conducted at two-week intervals following the first diet recall interview. A total of 10,307 dietary recalls were completed, with 96% of participants completing one dietary recall, 94% completing two dietary recalls, and 92% completing three dietary recalls. Details of the 24 hour dietary recall and post interview processing methodology employed have been described in detail in the VHM report (Department of Health, 2012b).

Interviewers were trained to assure competency and consistency in collected dietary recall information. Interviewers used a food model book to aid participants with their description of portion sizes of the food and beverages they had consumed. The food model book prompted dietary recall by including frequently forgotten foods and eating occasions, and assisted with

portion size estimation with 'to scale' photos of food and beverage containers, measuring spoons and cups (Department of Health, 2012b). All dietary recall interviews were supervised by certified dietitians from the Department of Nutrition and Dietetics, Monash University.

The FoodWorks® nutrition software (FoodWorks® software, FoodWorks Interview) was used to conduct the dietary recalls. A multiple-pass approach was used to assist participants to sufficiently recall their food and beverage intake. The software included a scripted guide for interviewers to help prompt participants for food recall during each interview. Interviewers were able to interrupt and prompt for further details on food items if required. Further information on the multiple-pass dietary recall process have been described in detail in the VHM report (Department of Health, 2012b).

On completion of the interviews, volume conversion factors were developed to convert food volumes into food weights. Conversion of food volume to weights were done by "reference to published data, by measuring the weight and volume of specific foods, or by considering the food as very similar to another food for which a volume conversion factor was already available" (Department of Health, 2012b). The AUSNUT 2007 (Food Standards Australia New Zealand, 2007) nutrient composition data were referred to calculate nutrient intakes based on estimated food intake. The mean intake for each nutrient was computed for each participant based on information collected from three 24 hour dietary recalls and was used in the analysis. This information was used to get a single measure of nutrient intake for each participant (Department of Health, 2012b).

3.2.5 Physical activity level[3]

The following criteria were used to define each participant's level of physical activity: sufficiently physically active (≥150 minutes of 'physical activity time' per week); and insufficiently physically active (0-149 minutes of 'physical activity time' per week) (Department of Health, 2012a). 'Physical activity time' was calculated as the sum of the time

spent walking or performing moderate activity plus double the time spent in vigorous physical activity (to reflect its greater intensity) (Armstrong, Bauman, & Davies, 2000).

3.2.6 Anthropometric measurements[3]

The anthropometric measurements included height, weight and WC, and these measurements were made at the testing sites by trained staff. Height was measured using a stadiometer, and participants were asked to remove their shoes. Weight was measured using a digital weighting scale, and participants were asked to wear light clothing and remove their shoes. Waist circumference was measured using a steel measuring tape. Further information on the anthropometric measurement methods for weight, height and WC have been previously described in the VHM report (Department of Health, 2012a).

3.2.7 Biomedical measurements[3]

Blood collection was conducted via venepuncture after an overnight fast of ten hours, or more. Blood samples were assessed for the following: total cholesterol, HDL-C, TG, HbA1c and FPG levels. Blood samples were centrifuged on site and were analysed at a separate central laboratory on a Siemens ADVIA 2400 Clinical Chemistry System (Siemens). Blood components were measured as following: total cholesterol using enzymatic (oxidase/peroxidase) methods; HDL-C using the elimination/catalase method; TG using GPO, Trinder with serum blank; blood glucose using the hexokinase method; and HbA1c was measured by immunoassay (Roche, Integra) (Department of Health, 2012a).

3.2.8 Blood pressure measurements[3]

Automated BP measurements (Dinamap, 8100, GE, USA) were made in all participants, in triplicate, while seated, after a five minute rest period. The average of the two closest measurements (<10 mmHg SBP and <6 mmHg DBP) were used in the analysis. Further details have been reported in the VHM report (Department of Health, 2012a).

3.3 Statistical analysis

3.3.1 Vitamin D

The primary dependent variable was serum 25OHD concentration, which was categorised into three groups based on its tertiles: low 25OHD tertiles (1st tertile: range 10-44 nmol/L; median 33nmol/L), medium 25OHD tertile (2nd tertile: range 45-64 nmol/L; median 54 nmol/L), and high 25OHD tertile (3rd tertile: range 65-204 nmol/L; median 77 nmol/L). We did not classify 25OHD into categories based on 'deficiency' or 'sufficiency' due to the lack of global consensus of cut-offs, and unbalanced sample sizes within each group.

3.3.2 Independent variables

To identify determinants of vitamin D status, we included a number of potential demographic, physical and lifestyle variables that could affect vitamin D status in the analysis based on the review of published evidence (Daly et al., 2012; Lips et al., 2014; Tsiaras & Weinstock, 2011), and also the completeness of information available in the VHM survey. The demographic variables were: age (18-34y, 35-44y, 45-54y, 55-64y, or 65-75y), gender (male, or female), country of birth (born in Australia, or born overseas), household income (<$30,000, $30,001-$70,000, ≥$70,001, or don't know/refused), education (high school or less, TAFE /diploma/certificate, or tertiary education), living area (metropolitan, or rural area), and season of biomedical assessment (summer, autumn, winter or spring).

The physical variables included BMI and the Modified Fitzpatrick Scale (MFS) for skin pigmentation. BMI was used as a categorical variable (non-obese <30 kg/m^2, or obese ≥30 kg/m^2). Skin pigmentation of participants were classified according to the MFS into five types, namely; dark brown or black skin colour (MFS score 4-5), brown skin colour (MFS score 6-7), light brown skin colour (MFS score 8-9), and fair skin colour (MFS score 10-12).

The lifestyle variables were smoking status (current smoker, or non-smoker/past smoker), physical activity (insufficient <150 mins/week, or sufficient ≥150 mins/week), total sitting

time per day (low <4h/d, moderate 4-8h/d, and high ≥8h/d), alcohol intake (low alcohol intake, medium alcohol intake, or high alcohol intake as standard drinks/d) and dietary calcium intake (low calcium intake, medium calcium intake, or high calcium intake as mg/d). Sitting time included activities such as visiting friends, driving, reading, watching TV, and working at a desk or a computer. Alcohol was classified as tertiles according to the number of standard drinks per day, with one standard drink equating to 10g of alcohol (National Health and Medical Research Council, 2009). Alcohol intake was classified as tertiles. Alcohol intake tertiles were: low alcohol intake (0 standard drinks/d), medium alcohol intake (<1-1.3 standard drinks/d) and high alcohol intake (>1.3-31 standard drinks/d). Dietary calcium intake were also classified based on its tertiles: low calcium tertile (1st tertile: range 72-719 mg/d; median 579 mg/d), medium calcium tertile (2nd tertile: range 720-1009 mg/d; median 858 mg/d), and high calcium tertile (3rd tertile: range 1010-3726 mg/d; median 1233 mg/d). We did not classify dietary calcium intakes based on the Australian RDI due to the small sample size within each age and gender category.

3.3.3 Statistical analysis

The statistical analysis was carried out step by step as given below:

Step 1: Demographic/descriptive statistics by the tertiles of 25OHD were obtained and differences between groups were tested by χ^2 test (or Fisher's exact test if applicable) for categorical variables of interest.

Step 2: Multinomial logistic regression analysis were used to examine the association between 25OHD tertiles and physical, demographic, and lifestyle determinants. Adjusted odds ratio (AOR) and 95% CI were obtained with the low (1st) tertile as the reference group. All the independent variables mentioned in Section 3.3.2 (age, gender, country of birth, smoking status, physical activity, income, education, season, BMI, MFS, living area, sitting time per day, alcohol intake and dietary calcium intake) were included initially in the regression analysis.

Step 3: A stepwise backward elimination regression method was then used to obtain the final parsimonious model, which only contained those variables which were associated with 25OHD tertiles at 5% significance level ($p<0.05$). In addition, selected two-way interaction effects of independent factors on 25OHD tertiles were investigated. The IBM SPSS Statistics for Windows, Version 21.0 'Complex Samples' module was used in the analysis to eliminate sampling bias arising from the multistage cluster sampling method used in the VHM survey.

3.4 Results

The study population contains of 46% males and 54% females with an average age of 49 y (SD=13.7). There were no significant differences in the concentration of vitamin D between males (55.4 nmol/L), and females (56.8 nmol/L, $p>0.05$). A larger proportion of the population were born in Australia (76%), were non-smokers (84%), were sufficiently physically active (68%), and were non-obese (75%). In this sample 41% had an education level of high school or less, 38% had a tertiary education, and 21% had a TAFE/diploma/certificate. As shown in Table 3.1, there were no significant differences across 25OHD tertiles according to age group ($p=0.195$), gender ($p=0.244$), income ($p=0.447$), education ($p=0.172$), smoking status ($p=0.272$) and residential location ($p=0.272$). A higher prevalence of people who were obese (41%, $p<0.001$), were born overseas (44%, $p<0.001$), were insufficiently physically active (41%, $p<0.001$), had dark brown or black skin colour (74%, $p<0.001$), had a greater sitting time (≥8h/d) (44%, $p<0.001$), had a biomedical examination in winter (46%, $p<0.001$), consumed a low amount of alcohol (0 standard drinks/d) (39%, $p=0.032$) and had low dietary calcium intake (1st tertile: range 72-719 mg/d; median 579 mg/d) (38%, $p=0.016$) were found to be in the low 25OHD tertile (range 10-44 nmol/L; median 33 nmol/L).

Table 3.1 Demographic characteristics of participants by vitamin D tertiles.

	Low 25OHD (33 nmol/L) (n=1,112)	Medium 25OHD (54 nmol/L) (n=1,163)	High 25OHD (77 nmol/L) (n=1,134)	P value
	N (%)	N (%)	N (%)	
Age (y)				0.195
18-34	217 (36%)	183 (30%)	209 (34%)	
35-44	247 (34%)	229 (32%)	242 (34%)	
45-54	285 (33%)	306 (36%)	269 (31%)	
55-64	213 (29%)	291 (39%)	243 (32%)	
65-75	142 (30%)	155 (32%)	178 (38%)	
BMI (kg/m^2)				<0.001
Non-obese (<30 kg/m^2)	794 (31%)	825 (32%)	935 (37%)	
Obese (≥30 kg/m^2)	346 (41%)	299 (35%)	202 (24%)	
Gender				
Males	559 (35%)	515 (33%)	510 (32%)	0.244
Females	573 (31%)	613 (34%)	639 (35%)	
Country of birth				<0.001
Born in Australia	771 (30%)	890 (34%)	937 (36%)	
Born overseas	360 (44%)	238 (30%)	213 (26%)	
Income				0.447
<$30,000	212 (437%)	172 (30%)	184 (33%)	
$30,001-$70,000	379 (36%)	359 (34%)	321 (30%)	
≥$70,001	488 (31%)	533 (34%)	570 (36%)	
Don't know/refused	64 (34%)	61 (32%)	66 (34%)	
Education				0.172
High school or less	434 (31%)	467 (34%)	481 (35%)	
TAFE/diploma/certificate	221 (31%)	226 (31%)	273 (38%)	
Tertiary education	478 (37%)	435 (33%)	394 (30%)	
Physical activity level (min/wk)				<0.001

	Low 25OHD (33 nmol/L) (n=1,112)	Medium 25OHD (54 nmol/L) (n=1,163)	High 25OHD (77 nmol/L) (n=1,134)	P value
Sufficient physical activity (≥150 min/wk)	700 (30%)	765 (33%)	850 (37%)	
Insufficient physical activity (<150 min/wk)	442 (41%)	358 (33%)	289 (26%)	
Smoking status				0.285
Smoker	202 (38%)	166 (31%)	165 (31%)	
Non-smoker	928 (32%)	963 (34%)	982 (34%)	
Modified Fitzpatrick Scale[a]				<0.001
Dark brown or black skin colour	70 (74%)	16 (16%)	9 (10%)	
Brown skin colour	264 (41%)	200 (31%)	181(28%)	
Light brown skin colour	318 (29%)	395 (35%)	402 (36%)	
Fair skin colour	437 (30%)	517 (34%)	544 (36%)	
Sitting time (h/d)				<0.001
Low: <4h /d	216 (26%)	301 (36%)	322 (38%)	
Moderate: 4-8h/d	557 (32%)	580 (33%)	610 (35%)	
High: ≥8h/d	357 (44%)	245 (30%)	215 (26%)	
Living in metro/rural				0.272
Rural	489 (28%)	627 (35%)	645 (37%)	
Metro	613 (37%)	516 (31%)	519 (32%)	
Season of biomedical examination[b]				<0.001
Summer	31 (15%)	74 (36%)	102 (49%)	
Autumn	119 (14%)	275 (34%)	415 (52%)	
Winter	530 (46%)	350 (31%)	267 (23%)	
Spring	504 (41%)	431 (34%)	311 (25%)	
Alcohol intake (standard drinks/d)				0.032
Low alcohol (0 standard drinks/d)[c]	423 (39%)	349 (32%)	321 (29%)	

	Low 25OHD (33 nmol/L) (n=1,112)	Medium 25OHD (54 nmol/L) (n=1,163)	High 25OHD (77 nmol/L) (n=1,134)	P value
Medium alcohol (0.5 standard drinks/d)[c]	330 (30%)	362 (33%)	401 (37%)	
High alcohol (2.6 standard drinks/d)[c]	340 (31%)	369 (34%)	384 (35%)	
Dietary calcium intake (mg/d)				0.016
Low calcium (579 mg/d)[c]	416 (38%)	365 (33%)	310 (29%)	
Medium calcium (858 mg/d)[c]	333 (30%)	333 (30%)	433 (40%)	
High calcium (1233 mg/d)[c]	347 (32%)	386 (35%)	356 (33%)	

Data are presented as mean estimate (weighted) (%) for variables. Difference in categorical variables between groups were assessed by Chi-square test.

Footnotes: BMI, body mass index; D day; h, hour; wk, week; [a], MFS score 4-5=dark brown or black skin colour; MFS score 6-7=brown or olive skin colour, MFS score 8-9=light brown or olive skin colour, MFS score 10-12=fair skin colour; [b], Summer=December-February; Autumn=March-May; Winter=June-August; Spring=September-November; [c], median of variable.

3.4.1 The association between 25OHD tertiles and physical, demographic, lifestyle and dietary factors

3.4.1.1 Initial model

Table 3.2 displays the results of the initial model, which revealed that those who were born overseas, were obese, were insufficiently physically active each week, were smokers, had a moderate to high sitting time, living in the metro area and biomedical examination in winter or spring had a lower odds of being in the medium or high 25OHD tertile.

Those who had a medium dietary calcium intake had a higher odds of being in either the medium or high 25OHD tertile. In addition, the models for age, MFS, and alcohol intake were not significant, however those who were 55-64 y, had light brown, or fair skin colour, and medium and high alcohol intake had higher odds of being in medium or high 25OHD tertile,

as compared with the reference category. There were no significant associations between 25OHD tertiles and gender, income and education.

Table 3.2 Initial model: The association between 25OHD tertiles and physical, demographic, and lifestyle factors.

	Low 25OHD (33 nmol/L)	Medium 25OHD (54 nmol/L)		High 25OHD (77 nmol/L)		P value
		OR	95% CI	OR	95% CI	
Gender						0.544
Female	Ref.					
Male		0.96	0.69, 1.33	0.85	0.62, 1.16	
Age (y)						0.060
18-34	Ref.					
35-44		1.01	0.72, 1.42	0.98	0.69, 1.40	
45-54		1.14	0.80, 1.64	0.88	0.63, 1.22	
55-64		1.53*	1.14, 2.04	1.83	0.87, 1.60	
65-75		1.24	0.87, 1.78	1.34	0.88, 2.04	
Country of birth						0.014
Born in Australia	Ref.					
Born overseas		0.70	0.47, 1.04	0.59*	0.42, 0.83	
BMI (kg/m^2)						<0.001
Non-obese (<30 kg/m^2)	Ref.					
Obese (≥30 kg/m^2)		0.62*	0.50, 0.76	0.36*	0.28, 0.46	
Physical activity (min/wk)						0.001
Sufficient (≥150min/wk)	Ref.					
Insufficient (<150min/wk)		0.72*	0.53, 0.99	0.48*	0.33, 0.68	
Smoking status						0.044
Non-smoker	Ref.					

	Low 25OHD (33 nmol/L)	Medium 25OHD (54 nmol/L)		High 25OHD (77 nmol/L)		P value
Smoker		0.74	0.54, 1.02	0.61*	0.39, 0.96	
Income						0.407
<$30,000	Ref.					
$30,001-$70,000		1.06	0.76, 1.47	1.03	0.61, 1.74	
≥$70,001		1.31	0.82, 2.10	1.45	0.92, 2.29	
Don't know/refused		1.07	0.55, 2.08	1.25	0.60, 2.60	
Education						0.32
High school or less	Ref.					
TAFE/diploma/certificate		0.91	0.60, 1.39	1.04	0.75, 1.44	
Tertiary education		0.89	0.54, 1.44	0.75	0.52, 1.08	
Modified Fitzpatrick Scale[a]						0.051
Dark brown or black skin colour	Ref.					
Brown skin colour		1.97	0.90, 4.31	2.30	0.90, 5.88	
Light skin colour		2.94*	1.31, 6.60	3.59*	1.38, 9.36	
Fair skin colour		2.64*	1.27, 5.52	3.30*	1.37, 7.92	
Sitting time (h/d)						<0.001
Low: <4h/d	Ref.					
Moderate: 4-8h/d		0.75*	0.57, 0.99	0.63*	0.50, 0.81	
High: ≥8h/d		0.46*	0.32, 0.68	0.32*	0.22, 0.47	
Living in metro/rural						0.031
Rural	Ref.					
Metro		0.64*	0.44, 0.93	0.53*	0.33, 0.84	
Season[b]						<0.001
Summer	Ref.					
Autumn		0.87	0.43, 1.75	0.80	0.30, 2.10	
Winter		0.24*	0.11, 0.53	0.10*	0.04, 0.28	
Spring		0.27*	0.11, 0.64	0.10*	0.03, 0.40	

	Low 25OHD (33 nmol/L)	Medium 25OHD (54 nmol/L)		High 25OHD (77 nmol/L)		P value
Alcohol intake (standard drinks/d)						0.126
Low alcohol (0 standard drinks/d)[c]	Ref.					
Medium alcohol (0.5 standard drinks/d)[c]		1.21	0.85, 1.72	1.46*	1.04, 2.04	
High alcohol (2.6 standard drinks/d)[c]		1.17	0.84, 1.63	1.51*	1.07, 2.13	
Dietary calcium intake (mg/d)						0.031
Low calcium (579 mg/d)[c]	Ref.					
Medium calcium (858 mg/d)[c]		1.13	0.87, 1.47	1.62*	1.20, 2.19	
High calcium (1233 mg/d)[c]		1.26	0.93, 1.72	1.35	0.86, 2.11	

All variables were adjusted for other variables in the model, which were: gender, age, country of birth, BMI, physical activity, smoking status, income, education, Modified Fitzpatrick Scale, sitting time, living in metro/rural, season, alcohol intake, and dietary calcium intake.

Footnotes: BMI, body mass index; D day; h, hour; Ref., reference category; wk, week; [a], MFS score 4-5=dark brown or black skin colour; MFS score 6-7=brown or olive skin colour, MFS score 8-9=light brown or olive skin colour, MFS score 10-12=fair skin colour; [b], Summer=December-February; Autumn=March-May; Winter=June-August; Spring=September-November; [c], median of variable;*, significant in comparison to reference group at 5% significance level.

3.4.1.2 Final model: Factors that reduced the odds of medium to high 25OHD tertiles vs. low 25OHD tertile

In the final model, those who were obese, were insufficiently physically active each week, were smokers, had a medium to high sitting time, living in metropolitan areas, and had their biomedical examination in winter or spring, had a lower odds of being in the medium or high

25OHD tertile. Obese (BMI ≥30 kg/m^2) participants were 36% and 63% less likely to have medium or high 25OHD (2nd and 3rd tertile) respectively vs. non-obese counterparts (BMI <30 kg/m^2) ($p<0.001$, Table 3.3).

Those who participated in insufficient physical activity (<150 min/wk) were 51% less likely to have a high 25OHD status compared to those who spent sufficient time on physical activity (≥150 min/wk) ($p<0.001$, Table 3.3).

Smokers had a 29% and 39% reduced odds of having a medium and high 25OHD respectively compared to non-smokers ($p=0.021$, Table 3.3).

As sitting time increased, the odds of having higher 25OHD status decreased. Those who sat for 4-8h/d had a 24%-37% reduced odds and those who sat for ≥8h/d had a 53%-68% reduced odds of having a medium or high 25OHD respectively vs. those sitting for <4h/d ($p<0.001$, Table 3.3).

Those residing in metro areas had a 36% and 47% reduced odds of medium and high 25OHD tertile respectively vs. their rural counterparts ($p=0.047$, Table 3.3).

Finally, participants whose biomedical examination was in winter had a 75% and 90% reduced odds of being in the medium and high 25OHD tertile compared to those measured in summer ($p<0.001$, Table 3.3). Similar outcomes were obtained for those measured in spring relative to those measured in summer (Table 3.3). There was no significant association between autumn and 25OHD tertiles.

3.4.1.3 Final model: Factors that increased the odds of medium to high 25OHD tertiles vs. low 25OHD tertile

The odds of having a medium to high 25OHD vs. low 25OHD were higher in those with medium dietary calcium intake. Those with medium dietary calcium intake had a 61% higher odds of high 25OHD vs. low dietary calcium intake ($p=0.026$, Table 3.3).

Table 3.3 Final model: The significant predictors of 25OHD status.

	Low 25OHD (33 nmol/L)		Medium 25OHD (54 nmol/L)		High 25OHD (77 nmol/L)		P value
	OR	95% CI	OR	95% CI	OR	95% CI	
Country of birth							0.124
Born in Australia	Ref.						
Born overseas			0.46	0.09, 2.28	0.50	0.08, 2.98	
BMI (kg/m^2)							<0.001
Non-obese (<30kg/m^2)	Ref.						
Obese (≥30 kg/m^2)			0.64*	0.51, 0.81	0.37*	0.29, 0.47	
Physical activity (min/wk)							<0.001
Sufficient (≥150mins/wk)	Ref.						
Insufficient (<150mins/wk)			0.74	0.54, 1.03	0.49*	0.35, 0.69	
Smoking status							0.021
Non-smoker	Ref.						
Smoker			0.71*	0.53, 0.95	0.61*	0.39, 0.93	
Modified Fitzpatrick Scale[a]							0.002
Dark brown or black skin colour	Ref.						
Brown skin colour			2.00	0.89, 4.52	2.37	0.70, 8.03	
Light brown skin colour			2.97*	1.05, 8.40	3.31	0.95, 11.50	
Fair skin colour			5.83*	2.35, 14.48	7.36*	2.34, 23.21	
Sitting time (h/d)							<0.001
Low: <4h/d	Ref.						
Moderate: 4-8h/d			0.76*	0.58, 0.99	0.63*	0.49, 0.80	
High: ≥8h/d			0.47*	0.33, 0.66	0.32*	0.22, 0.46	
Living in metro/rural							0.047
Rural	Ref.						

	Low 25OHD (33 nmol/L)	Medium 25OHD (54 nmol/L)		High 25OHD (77 nmol/L)		P value
Metro		0.64*	0.44, 0.95	0.53*	0.33, 0.87	
Season[b]						<0.001
Summer	Ref.					
Autumn		0.87	0.43, 1.77	0.82	0.32, 2.12	
Winter		0.25*	0.11, 0.52	0.10*	0.04, 0.29	
Spring		0.27*	0.11, 0.64	0.11*	0.03, 0.40	
Dietary calcium intake (mg/d)						0.026
Low calcium (579 mg/d)[c]	Ref.					
Medium calcium (858 mg/d)[c]		1.11	0.85, 1.45	1.61*	1.19, 2.16	
High calcium (1233 mg/d)[c]		1.19	0.88, 1.61	1.25	0.80, 1.96	

All variables were adjusted for other variables in the model, which were: country of birth, BMI, physical activity, smoking status, Modified Fitzpatrick Scale, sitting time, living in metro/rural, season, dietary calcium intake, country of birth x Modified Fitzpatrick Scale (interaction).

Footnotes: BMI, body mass index; D day; h, hour; Ref., reference category; wk, week; [a], MFS score 4-5=dark brown or black skin colour; MFS score 6-7=brown or olive skin colour, MFS score 8-9=light brown or olive skin colour, MFS score 10-12=fair skin colour; [b], Summer=December-February; Autumn=March-May; Winter=June-August; Spring=September-November; [b], median of variable; [c], median of variable;*, significant in comparison to reference group at 5% significance level.

3.4.1.4 Interactive model: The association between 25OHD tertiles and interaction between country of birth and MFS

Among all the potential two-way interaction effects tested, interactions between 'country of birth and BMI' (p=0.449), 'BMI and physical activity' (p=0.093), 'sitting time and BMI' (p=0.711), 'sitting time and physical activity' (p=0.553), 'MFS and season' (p=0.680), and 'country of birth and physical activity' (p=0.208) were found not significant. In the final

model, only the interaction between 'country of birth and MFS' was significant at 5% significance level (p=0.043), indicating the effect of MFS on 25OHD tertiles was modified by country of birth. In light of this effect modification, we performed separate regression analyses for participants who were born in Australia and those born overseas (Table 3.4-3.5).

The following interpretations will be focused on only the separate regression modelling of the significant interaction between country of birth and MFS (p=0.043) (Figure 3.1-3.2, Appendix P, Table P.1-P.2). For participants who were born in overseas (Figure 3.1, Appendix P, Table P.1), those with light brown skin colour, and fair skin colour had a higher odds of being in medium or high 25OHD tertile as compared with those with dark brown or black skin colour. The model for participants who were born in Australia (Figure 3.2, Appendix P, Table P.2), and MFS and 25OHD tertiles were not significant (p=0.296), however those with light brown skin colour had a higher odds of being in high 25OHD tertile as compared to dark brown or black skin colour.

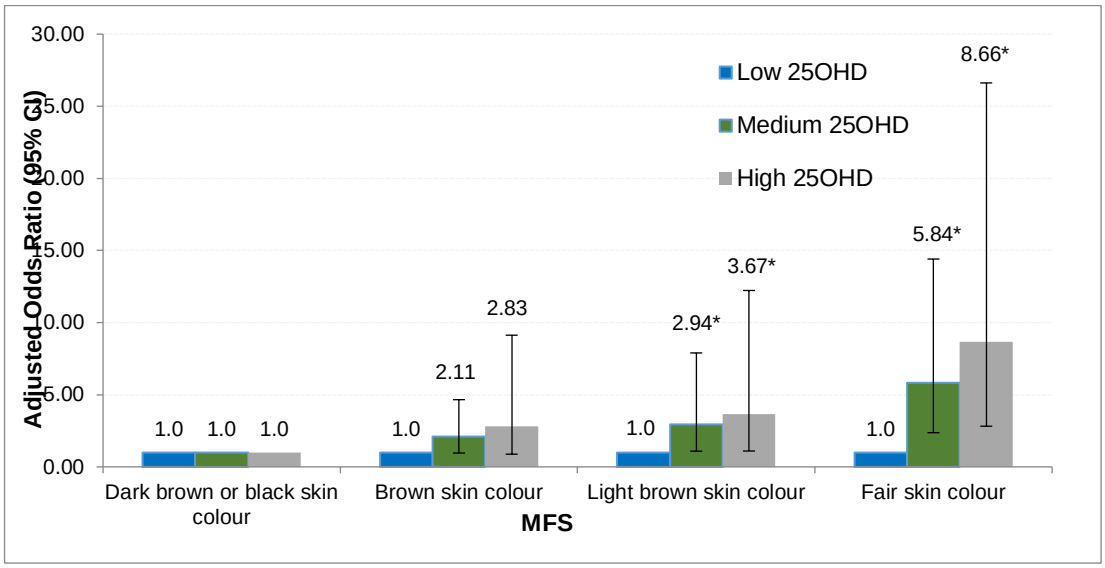

Figure 3.1 Interactive model of 25OHD tertile and MFS in those born overseas.

Model was adjusted for BMI, physical activity, smoking, sitting time, MFS, season, living area, dietary calcium intake.

Footnotes: Ref., reference category; *, significant in comparison to reference group at 5% significance level; [a], MFS score 4-5=dark brown or black skin colour; MFS score 6-7=brown skin colour, MFS score 8-9=light brown skin colour, MFS score 10-12=fair skin colour.

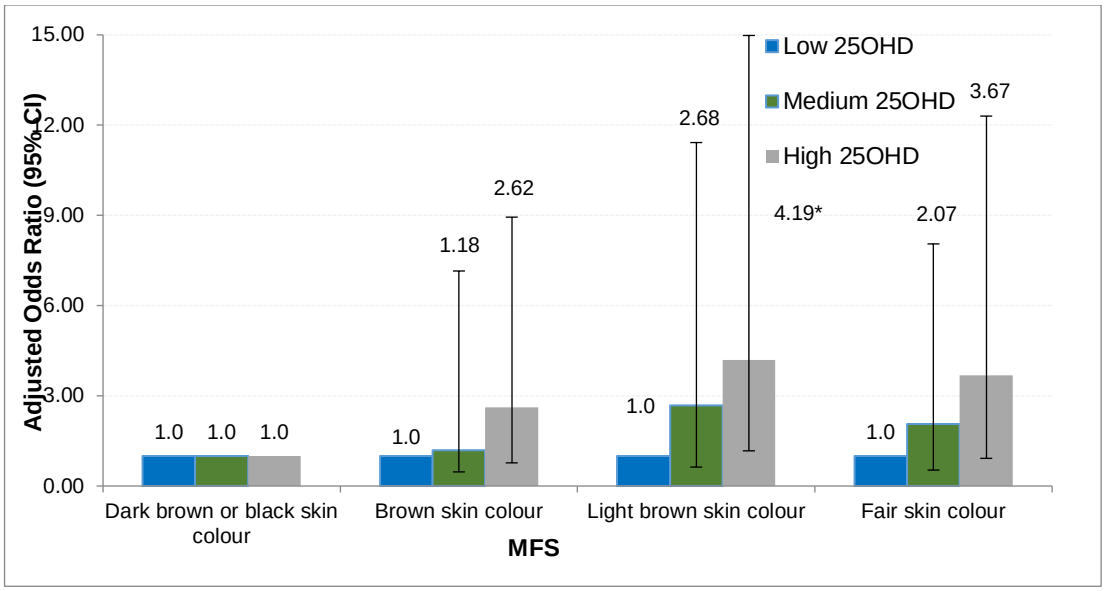

Figure 3.2 Interactive model of 25OHD tertile and MFS in those born in Australia.

Model was adjusted for BMI, physical activity, smoking, sitting time, MFS, season, living area, dietary calcium intake.

Footnotes: Ref., reference category; *, significant in comparison to reference group at 5% significance level; [a], MFS score 4-5=dark brown or black skin colour; MFS score 6-7=brown skin colour, MFS score 8-9=light brown skin colour, MFS score 10-12=fair skin colour.

3.5 Discussion

3.5.1 Obesity and 25OHD concentration

Over the last two decades, Australia has seen a dramatic rise in overweight and obesity rates, with approximately 28% of adult males and 30% of females being obese (Ng et al., 2014). In this study we found that those who were obese (BMI ≥30 kg/m^2) were less likely to have a higher 25OHD concentration as compared to their non-obese counterparts. This confirms the well documented inverse relationship between adiposity and 25OHD levels (Brock et al., 2010; Burgaz, Akesson, Oster, Michaëlsson, & Wolk, 2007; Saneei et al., 2013;

Scragg & Camardo Jr., 2008; Vimaleswaran et al., 2013). The reasoning for this inverse relationship however has not been established conclusively, though some potential explanations exist. First, 25OHD is a fat soluble vitamin, and the major storage site in the body is adipose tissue (Rosenstreich et al., 1971). The term 'sequestration' was introduced by Wortsman et al. (2000) who suggested that 25OHD disappears into adipose tissue and is not available for metabolic activity. This would imply that obese individuals have lower 25OHD concentration due to the sequestration of 25OHD in adipose tissue. A second explanation favoured by Drincic et al. (2012) is a volumetric dilution effect. This suggests that a greater body size and adipose tissue stores in obese individuals results in a lower plasma 25OHD level. This may mean that with weight loss 25OHD may potentially return to the plasma and levels may increase. We have investigated this hypothesis as part of this thesis [Chapter 4]. However, other mechanisms such as the conversion of 25OHD to inactive metabolites, the paracrine use of 25OHD in adipose tissue and other mechanisms need to be further investigated. However, inter-related with obesity may also be decreased levels of sun exposure due to limited mobility and increased sitting time, as well as limited physical activity.

Conversely, whether low vitamin D levels may lead to obesity has also been questioned. Foss (2009) describes the potential physiological processes that outlines vitamin D as being a casual factor in obesity. Low vitamin D levels may trigger an 'adaptive winter response' resulting in an accumulation of fat mass. However, a recent large mendelian randomisation study discounted these findings and did not find any evidence to support a causal role of vitamin D in the development of obesity (Vimaleswaran et al., 2013). Other data from our laboratory have also found that vitamin D supplementation in the absence of weight loss has no effect on body fat mobilisation (Pathak et al., 2014). Adipose tissue is a dynamic endocrine organ, and may modulate and be regulated by vitamin D (Earthman, Beckman, Masodkar, & Sibley, 2012). Adipose tissue also secretes adipokines such as leptin (Matsuzawa, Funahashi, Kihara, & Shimomura, 2004; Weyer et al., 2001). Leptin may affect vitamin D synthesis via negatively affecting the pathway of 1-alpha hydroxylase in certain tissues, resulting in lower

levels of the active metabolite (1,25-dihydroxyvitamin D) of vitamin D (Maetani, Maskarinec, Franke, & Cooney, 2009; Menendez et al., 2001).

3.5.2 Insufficient amounts of physical activity and 25OHD concentration

Higher intensity and frequency of physical activity tends to be linked to higher 25OHD status (Bertrand et al., 2012; Millen et al., 2010; Scragg & Camardo Jr., 2008). Consistent with this, we found that those who were insufficiently active tended to have a lower odds of having a higher 25OHD status. This may be explained by limited sun exposure in those who exercise minimally, or limited skeletal muscle activity which may influence the metabolism of vitamin D (Scott et al., 2010). One study found that outdoor activity and the frequency of physical activity were related to 25OHD status, rather than indoor activity and intensity of the exercise (Scragg & Camardo Jr., 2008). In contrast, another study found that outdoor and indoor physical activity were both significantly associated with 25OHD. Gardening and cycling were strongly associated with 25OHD whereas outdoor walking was not (van den Heuvel, van Schoor, de Jongh, Visser, & Lips, 2013). This may suggest that the association between 25OHD and gardening and cycling may be due to the higher intensity of these activities, which has also been identified by others (Bertrand et al., 2012; Hibler et al., 2016; Millen et al., 2010). 25OHD status has been found to be linked with greater muscle building exercises (Bell, Godsen, Henry, Shary, & Epstein, 1988), greater muscle mass, and strength (Scott et al., 2010). Thus those who are insufficiently active leading to muscle inactivity, have lower levels of muscle mass (Jones et al., 2004; Ringholm et al., 2011) and loss of muscle strength (Jones et al., 2004; Morris & Jacques, 2013) thus a decreased ability to metabolise 25OHD. Further studies into the intensity and types of physical activity, and its effects on 25OHD and its metabolites would be beneficial.

3.5.3 Increased sitting time per day and 25OHD concentration

We found that an increase in sitting time from moderate (4-8h/d) to high (≥8h/d) decreased the odds of higher 25OHD status, as compared to low sitting time (<4h/d). This outcome was independent of physical activity as described above. Few studies have investigated and identified that increased sedentary time equates to a lowered vitamin D status (Brock et al., 2010; Thuesen et al., 2012), however one recent study found no association between sedentary behaviours and the circulating vitamin D metabolite ($1,25(OH)_2D$) or 25OHD levels (Hibler et al., 2016). Though other studies have indicated a link with increased television viewing time and 25OHD status (Daly et al., 2012; Hypponen, Berry, Cortina-Borja, & Power, 2010). Recently, the number of sitting hours per day has received much attention as it has been linked with increased risk of CVD, myocardial infarction and all-cause mortality (Katzmarzyk, Church, Craig, & Bouchard, 2009; Petersen et al., 2014). Low 25OHD levels are also linked with higher risk of CVD, MetS and T2DM. It could be postulated, that those with greatest sitting time per day coupled with low 25OHD levels may potentially be at greater risk of metabolic disease, than those who sit less with adequate 25OHD levels.

3.5.4 Country of birth, MFS and 25OHD concentration

Country of birth may be an indicator of ethnicity, which may be used as a marker for skin pigmentation. In this sample approximately 24% were born overseas and had a lower odds of a higher 25OHD status as compared to those born in Australia. The MFS indicated that those who were born overseas with skin colour ranging from fair, light brown and brown had two to five times greater odds of higher 25OHD status than those with dark brown or black skin colour. This is due to greater skin pigmentation acting as a barrier against solar UVB photons that infiltrate the skin, thus limiting the amount of vitamin D_3 produced in the skin (Clemens et al., 1982). This is in line with other evidence that has shown that darker skin individuals tend to have lower 25OHD levels than fair skinned individuals (Harris, 2006; Hirani, Mosdøl, & Mishra, 2009). Conversely, a few studies have now found that those with more pigmented skin have the ability to produce similar amounts of cutaneous vitamin D to their light skinned

counterparts, after prolonged exposure to UVB light (Clemens et al., 1982; Lo, Paris, & Holick, 1986). It may be possible that those who are fair skinned and born overseas, have increased sun exposure in Australia compared to their parent country. Other factors relating to country of birth may include clothing practices and sun seeking behaviour. The amount of clothing worn, may be for religious reasons such as the hijab or niqab, which may cover all parts of a woman's body including the face, thus limiting cutaneous synthesis of vitamin D (Mithal et al., 2009). Those residing in Australia, who wear the hijab tended to have lower levels of 25OHD (Diamond, Levy, Smith, & Day, 2002; Grover & Morley, 2001). Clothing practices may also differ due to cultural reasons where those from south Asia tend to cover up with clothing more than these from Europe, and America (Islam, Akhtaruzzaman, & Lamberg-Allardt, 2006). Some differences may also lie in the sun seeking behaviour of different ethnic groups, with the avoidance of sun due to the appeal of fair skin in certain cultural groups (Mithal et al., 2009). Australia has a well-developed program of safe sun exposure that is communicated widely to the population. Hence uptake of such messages of increased use of sun screen, sun protection clothing and seeking shade in hot climates would lead to decreased sun exposure (Faurschou et al., 2012; Jayaratne, Russell, & van der Pols, 2012) and possibly explain why Australian born do not show the expected variation due to MFS as seen with those born overseas (Table 3.5).

There are some limitations to these arguments. Skin colour was classified according to a self-administered MFS, thus there may be inaccuracies in the way that individuals categorised their skin colours according to personal judgement (Eilers et al., 2013). The differences in VDR gene polymorphisms between racial groups may explain differences in 25OHD, with an association between certain VDR genotypes and low 25OHD levels which were found in Egyptian and Turkish (Baroncelli et al., 2008) subjects but not Lebanese (Arabi, Mahfoud, Zahed, El-Onsi, & El-Hajj Fuleihan, 2010). We did not collect data on how long those who were born overseas have resided in Australia. The inclusion of this factor could prove or disprove the story of Australian public health messages. Figure 3.2 appears to show a trend

between MFS and those who were born in Australia, which appears to be amplified in those born overseas. A larger sample size may have shown a greater effect in MFS, those born in Australia and 25OHD. Lastly, poorer sun protection behaviour (Hay, Coups, Ford, & DiBonaventura, 2009) in those born overseas may also explain higher 25OHD status.

3.5.5 Season and 25OHD concentration

The amount of vitamin D_3 synthesised in the skin is reliant on UVB radiation. The amount of solar radiation is dependent on latitude as well as season (Mithal et al., 2009). Similar to other studies (Brock et al., 2010; Hirani et al., 2009; Poopedi, Norris, & Pettifor, 2011; Thuesen et al., 2012), we found a marked seasonal fluctuation in 25OHD levels. We observed that the odds of having higher 25OHD were lowest during winter and spring, with the odds being reduced in autumn. The literature also indicates that 25OHD status is lowest during winter for all ages across the world (Mithal et al., 2009). This may be due to a lack of time spent outdoors coupled with increased skin or coverage, or a lack of UVB exposure. In a southern state such as Victoria, the greater distance from the equator and decreasing UV radiation (UVR) would also play a part in the lower odds of achieving a higher 25OHD status, especially during winter as compared to countries closer to the equator.

3.5.6 Dietary calcium intake and 25OHD concentration

Vitamin D stimulates calcium absorption from the gut and tightly controls bone mineralisation (Lips et al., 2014), thus the association between the two nutrients appears logical. Higher calcium intake inhibits PTH activity and reduces serum PTH. This in turn reduces the production of vitamin D metabolites. An early study in rats found that high calcium intakes increased 25OHD, however low calcium intake heightened the metabolic clearance of 25OHD (Clements, 1987). A recent study in rats found that low dietary calcium intake reduced 25OHD levels, regardless of an additional vitamin D supplement (Anderson et al., 2010). Whereas higher dietary calcium intakes increased 25OHD levels, and also increased the half-life of 25OHD, compared to those on a low calcium diet. This is consistent with a clinical

study that showed primary or secondary hyperparathyroidism reduced the half-life of 25OHD. This is just one explanation of the potential mechanisms between 25OHD and calcium, however there may be other processes involved.

It is interesting to note from this study that medium calcium intake (~858 mg/d) appears to be most beneficial to have higher 25OHD status, rather than high calcium intake (1,233 mg/d) with the latter being closer to the RDI for adult calcium intake (1,000-1,300 mg/d). However the interplay between calcium intake and absorption and 25OHD is complex (Heaney, 2008). There appears to be a potential plateau between the amounts of calcium absorbed according to 25OHD status (Heaney, 2008). As 25OHD approaches 80 nmol/L the absorptive efficiency increases, however above this point there is no further action on the absorption of calcium. Regardless of the mechanism, the role of dietary calcium and 25OHD beyond bone is under-appreciated and its beneficial effects need further investigation.

3.5.7 Strengths

To the best of our knowledge our study had one of the larger sample sizes, as compared to other studies worldwide (BinSaeed et al., 2015; Brodie et al., 2013; Chailurkit, Aekplakorn, & Ongphiphadhanakul, 2011; Freedman et al., 2013; Kimlin et al., 2014; Tonnesen, Hovind, Jensen, & Schwarz, 2016; Touvier et al., 2015). Aside from a study by Daly et al. (2012) which consisted of a national sample (n=11,247), ours was the next largest sample in Australia. In our study, blood samples measuring vitamin D concentration were conducted in one laboratory, which may improve accuracy and reliability of the measurement. Differences in vitamin D concentration have been found when measuring samples in different laboratories using the same assay (Black et al., 2015). Calcium intake is tightly inter-related with vitamin D concentration, and our study appears to be the first to include this variable as a potential determinant of vitamin D concentration. We and one other study (Daly et al., 2012) found sitting time to be a determinant of vitamin D concentration, however others had not explored this variable (BinSaeed et al., 2015; Brodie et al., 2013; Chailurkit et al., 2011; Freedman et

al., 2013; Kimlin et al., 2014; Thuesen et al., 2012; Tonnesen et al., 2016; Touvier et al., 2015). We were also able to explore MFS, however others did not have this data (BinSaeed et al., 2015; Brodie et al., 2013; Freedman et al., 2013; Kimlin et al., 2014; Thuesen et al., 2012; Tonnesen et al., 2016).

3.5.8 Limitations

Our study may be limited in not measuring sun exposure or the amount of clothing worn, as they are two influential factors in synthesising vitamin D (Kimlin et al., 2014). We have adjusted our models for season of blood collection, though this does not substitute as an assessment for sun exposure. Pregnant women tend to be at higher risk of vitamin D deficiency (Lips et al., 2014), however this data was not collected. A more accurate measure of categorising skin pigmentation, rather than self-categorising, may be through dermatologist determined Fitzpatrick skin phenotype via spectrophotometry (Eilers et al., 2013). There appears to be a trend between MFS, those born in Australia and 25OHD (Figure 3.2), however a significant association was not identified thus may be a limitation in this analysis. Genetic variation may affect vitamin D concentration where certain alleles may increase the risk of vitamin D insufficiency, however this data was not collected (Wang et al., 2010). The VHM survey did not collect information on vitamin D supplement usage, or foods that were fortified with vitamin D, thus the contribution of each on 25OHD could not be teased out.

3.6 Conclusion

In conclusion, we found that those who were obese, participated in insufficient physical activity, were born overseas and had darker skin had a lower odds of higher 25OHD levels. Our study is unique as we were able to classify individuals based on the Modified Fitzpatrick Scale, which is the gold standard for classifying skin pigmentation (Roberts, 2009), whereas other studies have not used any skin criteria (Alkerwi et al., 2012; Daly et al., 2012). During the cooler months, there was a lower odds for higher 25OHD, most probably due to less time spent outdoors, increased skin cover for winter and lower UVR. We also found that those who

spent more time engaged in sitting activities had a lower odds of higher 25OHD than those who spent less time sitting. A unique finding in this study was that those who were born overseas with light brown, fair skin colour tended to have a greater odds of a higher 25OHD tertile than those with darker skin. This was not observed in Australian born. This is an interesting finding that requires further investigation. Overall, 25OHD may be determined by a number of physical, socio-demographic and lifestyle factors. Inclusion of genetic determinants would provide further insight into vitamin D metabolism, and factors that may affect circulating concentration. These determinants should influence future strategies aimed at improving the 25OHD status of Australians.

Chapter 4 Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects

The content of this chapter is covered by a published paper (Appendix B).
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Pannu, P. K., Zhao, Y., & Soares, M. J. (2016). Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects: a systematic review and metaregression analysis. *Nutrition Research, 36*(3), 201-213. doi:10.1016/j.nutres.2015.11.013

Objective addressed

Objective 3: To investigate the effect of weight loss on vitamin D status in obese subjects.

4.1 Introduction

Vitamin D and PTH are essential for calcium homeostasis and bone metabolism (Ross et al., 2011). There is accumulating evidence that vitamin D plays an important role in extra-skeletal health and diseases such as diabetes mellitus, cancers, CVD and autoimmune disorders (Hollick, 2004; Parker et al., 2010; Pilz et al., 2013; Vimaleswaran et al., 2014). 25OHD is the best clinical indicator of vitamin D status (Ross et al., 2011), and based on current cut-offs, the prevalence of vitamin D insufficiency worldwide is high. The escalating obesity crisis potentially contributes to this increasing incidence of vitamin D insufficiency, since obese individuals have lower levels of 25OHD than their non-obese counterparts (Lagunova, Porojnicu, Lindberg, Hexeberg, & Moan, 2009; Mai et al., 2012; Samuel & Borrell, 2014). In fact inverse associations between body weight, BMI or measures of body fatness with vitamin D status has been found across the lifespan (Earthman et al., 2012; Samuel & Borrell, 2014; Soares et al., 2011a; Vimaleswaran et al., 2014). Differences in 25OHD levels can be attributed to age, race, geography, skin colour, habitual clothing and sun exposure amongst other factors (Mason, Sequeira, & Gordon-Thomson, 2011b). However as vitamin D is fat soluble, it is commonly considered that the lower levels in the obese could also be due to uptake by adipose tissue and its clearance from plasma.

Rosenstreich et al. (1971) were the first to propose that adipose tissue was the major storage site of vitamin D, and that its release from this tissue was quite slow. Based on the available evidence from animals and man, Heaney et al. (2009) have confirmed that distribution of 25OHD was highest in fat mass (FM) (34%), followed by serum (30%) and then muscle (20%). Wortsman et al. (2000) instead referred to ‘sequestration’ of 25OHD for their observation that UVB radiation resulted in a significant increase in serum vitamin D_3 in non-obese compared to obese individuals. This implied that vitamin D ‘disappeared’ into adipose tissue and other tissues and was not immediately available in plasma for further

metabolic activity. Such a phenomenon would account for the lower bio-availability of the vitamin in the obese (Wortsman et al., 2000), however, the mechanisms controlling the deposition and release of vitamin D from adipose tissue are still unknown (Malmberg et al., 2014).

Drincic et al. (2012) however support the theory of volumetric dilution, which implies that plasma levels of the vitamin decrease as body size and hence fat stores increase. It follows that if fat stores decrease, there ought to be a greater return of vitamin D into plasma resulting in increased vitamin D status. In a cross-sectional study, Drincic et al. (2012) identified body weight as the single strongest predictor of 25OHD levels, followed by FM. Their best fitting model relating 25OHD and body weight was a hyperbola, which indicated that body weight explained 13% of the variance in 25OHD. A visual inspection of the regression line shows that the slope is steeper at a body weight <90 kg but gets progressively shallower at higher body weights (Drincic et al., 2012). Hence an obese individual of 100 kg would need to lose a considerable amount weight to benefit from an appreciable increase in 25OHD. The results of a clinical trial would support this interpretation since categories of weight loss <15% of baseline brought about increases of 5.3-8.3 nmol/L in 25OHD, while above a value of 15%, there was more than a doubling of this effect (Mason et al., 2011a). A caveat to such expectations would be the extensive conversion of released vitamin D to metabolites other than 25OHD, which would not be detected by the specific 25OHD assay used (Figure 4.1).

It is unclear how 25OHD is handled once taken up by different body tissues like adipose tissue and skeletal muscle. Both tissues are metabolically active and the VDR is expressed in them (Ding, Gao, Wilding, Trayhurn, & Bing, 2012; Pfeifer, Begerow, & Minne, 2002). Hence a paracrine role in these tissues may account for some of the sequestration effect. Alternatively if these tissues merely act as a store for the vitamin, then a sizeable amount would be available for release into plasma following tissue mobilisation in response to weight loss (Mawer, Blackhouse, Holamn, Lumb, & Stanbury, 1972). There is also the possibility that both sequestration and volumetric dilution co-exist in obese individuals. In Figure 4.1 we

schematically depict the basis of this review and the potential storage and release of 25OHD in an obese individual during weight loss. We focused on the larger stores of adipose tissue seen in overweight/obese individuals to allow for the best chance for hypothesised effects. We also negated the contribution from external sources of vitamin D by excluding study arms with vitamin D supplementation, and those that reported excessive sunlight exposure during their trials. In this systematic review we embarked on the hypothesis that weight loss without supplementary vitamin D would result in an increase in plasma 25OHD. We entertained the possibility that changes in 25OHD might be explained by either volumetric dilution effect, sequestration effects as well as other mechanisms (Figure 4.1).

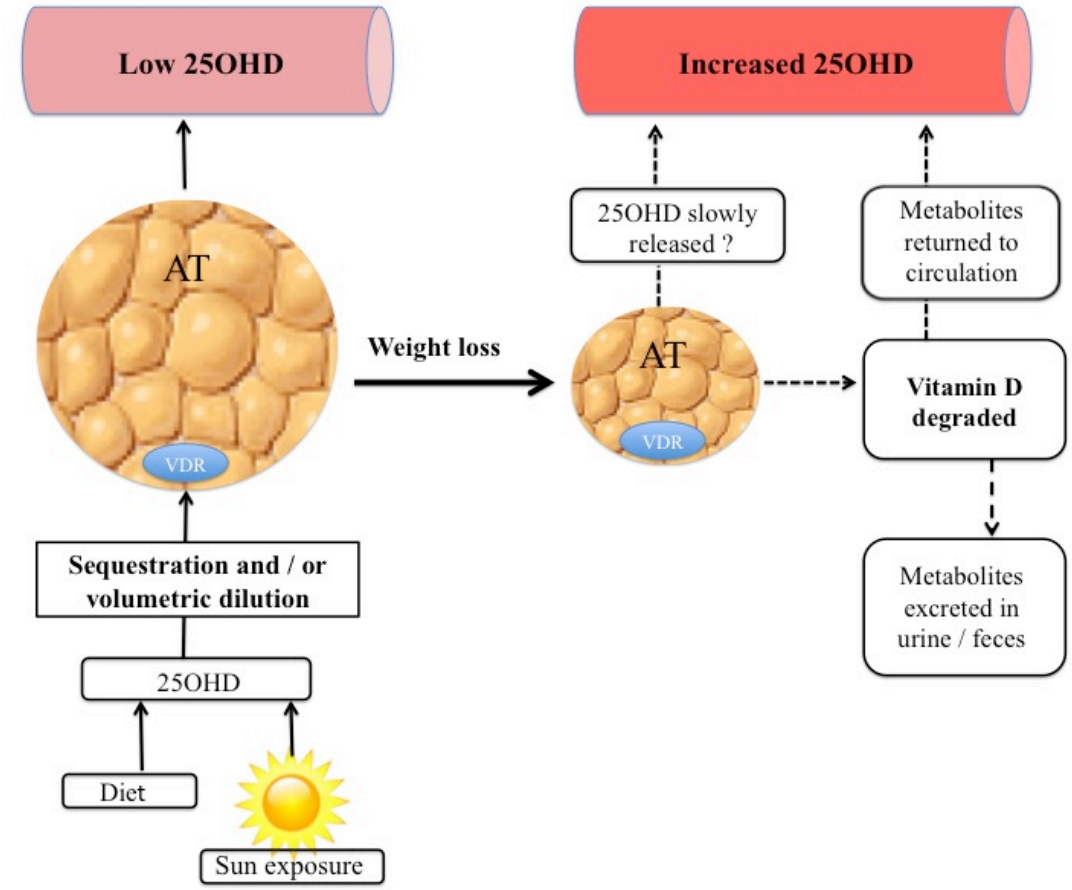

Figure 4.1 The potential pathways influencing 25OHD in obese individuals before and after weight loss.

4.1.1 Systematic review

The aim of the search was to identify trials with weight loss that measured change in vitamin D status, but without vitamin D supplementation. Accordingly placebo arms of trials that used vitamin D supplementation were included since we were only interested in relating the change in the two variables. Studies were identified through a systematic electronic search of Web of Science and PubMed Central databases over the period January 1994 to October 2015. One author (PKP) conducted the search using the following terms: vitamin D, vitamin D-3, 25-hydroxy-vitamin D, 25-hydroxyvitamin D, serum 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D3, 25OHD, cholecalciferol, 25-OH vitamin D, 25-hydroxycholecalciferol or serum 25OHD, and obese, overweight, caloric restriction, weight loss, fat mass, fat free mass, body mass index, BMI or adipose tissue. Only articles published in the English language were included.

At the identification stage the abstract was read, and the articles were selected, according to the following inclusion criteria: human clinical trials, weight loss study (through caloric restriction, increased physical activity or both), measurement of weight loss or body composition, study or placebo arm/s without vitamin D supplementation, overweight/obese subjects and change in serum 25OHD. Exclusion criteria included the use of the following terms in the abstract: vitamin D supplementation in all study arms, vitamin D enriched foods greater than >800I U/d, animal studies, gastric bypass/bariatric surgery studies, and duplicates of the same article retrieved from the two different databases. At the screening stage, the full text was read and articles were screened based on the following inclusion criteria: change in serum 25OHD measured, included data for at least one index of weight change, and weight loss as the primary or secondary outcome. Articles were excluded if: vitamin D supplements were used, diets included foods enriched with vitamin D to result in >800I U/d or extreme exposure to sunlight was indicated. Additional studies were sourced by manually searching the reference list, and included two published studies from our laboratory (Chan She Ping-Delfos, 2009; Cummings, 2006). After eligibility was determined, all RCTs were graded for

their quality according to the Jadad score, with values ≥3, indicating a high quality study (Jadad et al., 1996), while nine single stranded studies were graded as zero. The overall process is outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocols (PRISMA) (Moher et al., 2015) flow diagram (Figure 4.2).

Data extraction was carried out by one investigator (PKP) on an excel spreadsheet developed by the statistician (YZ). Another investigator (MJS) crossed checked quality criteria assessment and data entry. Any discrepancies were reviewed and discussed. The change in mean and standard deviation was calculated for body weight, FM, FFM or BMI, for studies that provided only pre and post values. Where necessary, vitamin D intake data were converted to IU/d and 25OHD status to nmol/L. FM was extracted as a percentage and FFM as kg as the majority of articles presented their data in this manner. All subjects were overweight or obese at baseline thus FM (%) is an appropriate measure during weight loss studies. Furthermore, it is common to use FM (%) and FFM (kg) to evaluate nutrition status (Kyle, Schutz, Dupertuis, & Pichard, 2003).

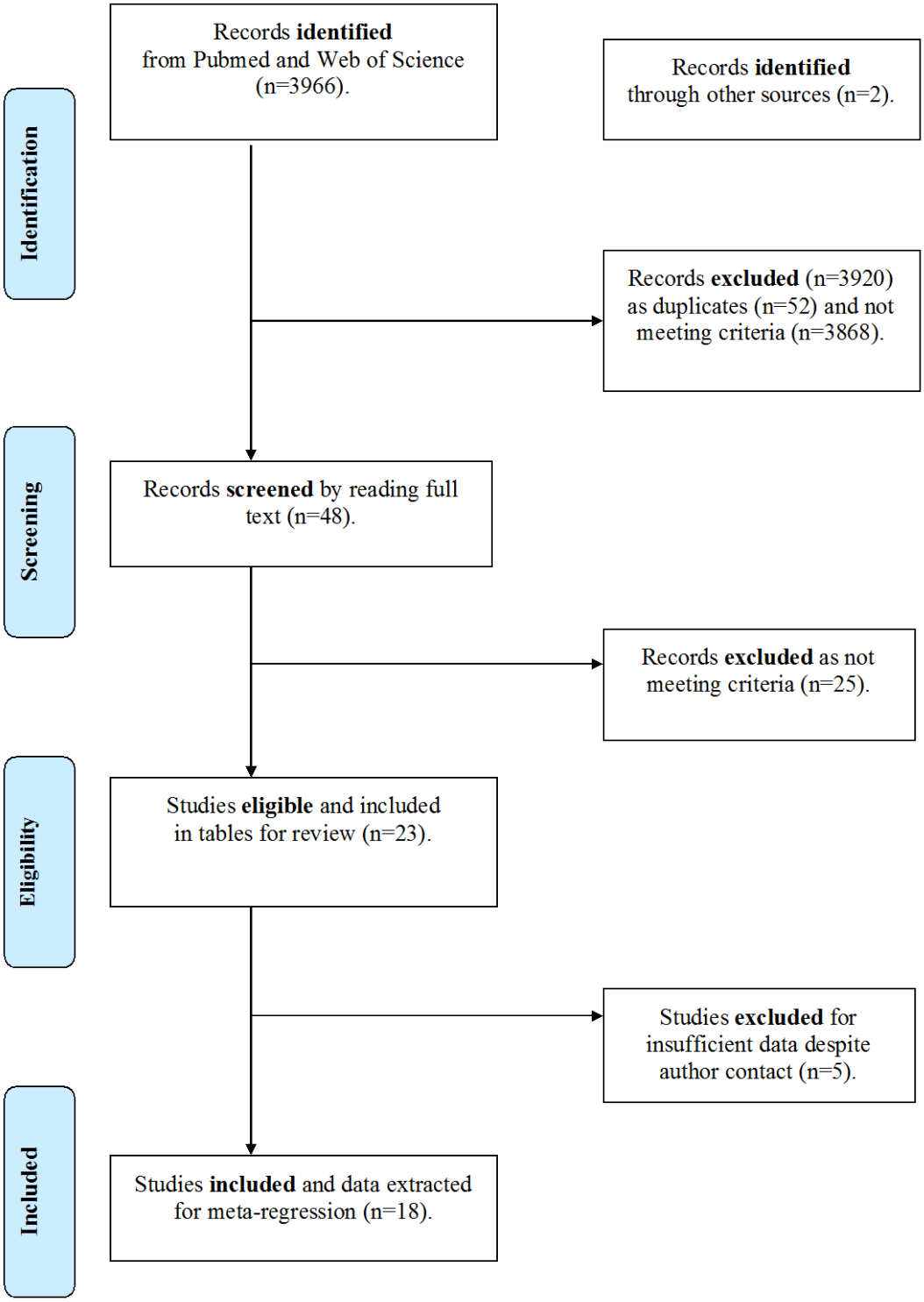

Figure 4.2 PRISMA flow diagram for vitamin D status and weight loss.

(Moher et al., 2015)

4.2 Methods and materials

4.2.1 Statistical analysis

4.2.1.1 Meta-analysis main effects

The primary aim was the relationship of change in vitamin D status and change in weight/obesity status. The change in vitamin D status was calculated as post-value minus the pre-value where a positive value implied an increase in the 25OHD status. Changes in the four main factors of interest in our paper, i) weight (kg); ii) BMI (kg/m^2); iii) FM (%); and iv) FFM (kg), were also calculated as post-value minus the pre-value; hence a negative value implied a reduction in the four main factors. Some RCTs had multiple treatment arms. Each arm was included as a separate study in the meta-analysis. Both fixed-effect and random-effects meta-analysis models were carried out to obtain the weighted mean difference (WMD) of vitamin D status based on the studies included, in order to extrapolate results to the general population. To test for heterogeneity and identify the potential sources of heterogeneity, I^2 statistics and Galbraith plot were used. Potential publication and small sample size bias was assessed by visual inspections of funnel plots and Egger's test.

4.2.1.2 Confounders

Potential confounders considered for analyses were mean age of subjects, percentage of females in each trial, duration of trial, vitamin content in food (IU/d), total vitamin D intake in trial (IU/d x duration of trial) and season (Pathak et al., 2014; Zheng et al., 2013). In our experience age and gender (% female) are potential confounders as they worked in opposite directions in regards to the effect of vitamin D supplementation on body weight (Pathak et al., 2014). In this paper, variations in 25OHD between gender may be due to differences in body composition, with women having a higher percentage of FM (Bolland et al., 2007; Lagunova et al., 2009). Duration of intervention may make a contribution to 25OHD, since greater weight loss can be expected over a longer intervention period (Finkler, Heymsfield, & St-Onge, 2012). An effect of season is potentially inter-twined with duration of study. Weight loss

studies commencing in winter and lasting over summer may result in an increase in 25OHD due to season that will confound the expected increase due to greater weight loss (Daly et al., 2012). However, we could not control for season as this was stated in only four studies (Cummings, 2006; Ibero-Baraibar, Navas-Carretero, Abete, Martinez, & Zulet, 2015; Lucey et al., 2008; Ortega et al., 2009). The trials included in this review had also employed a range of assays to measure 25OHD. These included radioimmunoassay (RIA) (n=12), chemiluminescence immune assays (CLIA) (n=4), competitive protein binding assays using rachitic rat kidney cytosol (CBA) (n=1), enzyme-linked immune absorbent assay (ELISA) (n=1), electrochemiluminescent immunoassay (ECLIA) (n=1), liquid chromatography mass spectrometry (LC-MS) (n=2), and not reported (n=2) (Table 4.1). Potential control for assays was considered, but were not carried out due to the limited number of high quality studies using the same technique (RIA n=6, CLIA n=2, ELISA n=1). Body composition techniques also varied, including: DEXA (n=12), BIA (n=6), skinfold thickness measurements and equations (n=2), and three studies did not report their method. Due to the variation in body composition techniques used and the limited number of high quality studies using the same technique (DEXA n=6, BIA n=1), no sub-group analysis was attempted.

4.2.2 Meta-regression analysis

Separate unadjusted and adjusted random-effects meta-regression analyses were carried out to investigate the independent contribution of the changes in the four main factors on the WMD of vitamin D status. A restricted maximum likelihood (REML) estimation method with backward elimination regression procedure was used in the meta-regression analyses. Bubble plot was obtained with the size of the "bubble" proportional to the precision of the estimate for each of the four individual factors. The meta-regression analyses were conducted in three steps: 1) unadjusted, 2) adjusted for study quality where a Jadad score was treated as a categorical variable ($0 < 3$, $1 \geq 3$) and 3) further adjustment for age, percentage of females, duration, and vitamin D in study diets (IU/d). All data analyses were carried out by Stata

version 12 (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP). A p value of less than 0.05 was considered as statistically significant.

4.3 Results

4.3.1 Systematic review

The search strategy generated 23 studies (14 RCTs and 9 single stranded studies) whose key features are presented in Table 4.1. Of these studies, twelve were conducted in Europe (Christensen et al., 2012; Damms-Machado, Weser, & Bischoff, 2012; Holecki et al., 2007; Holecki et al., 2008; Ibero-Baraibar et al., 2015; Jensen, Kollerup, Quaade, & Sorensen, 2001; Lucey et al., 2008; Ortega et al., 2009; Reinehr, de Sousa, Alexy, Kersting, & Andler, 2007; Tzotzas et al., 2010; Wamberg et al., 2013; Zittermann et al., 2009), five in USA (Apovian et al., 2009; Mason et al., 2011a; Ricci et al., 1998; Riedt et al., 2007; Van Loan et al., 2011), two in Canada (Gangloff et al., 2015; Josse, Atkinson, Tarnopolsky, & Phillips, 2011), two in Australia (Chan She Ping-Delfos, 2009; Cummings, 2006) and two in the Middle East (Albadah et al., 2015; Shahar et al., 2010). The study settings were all outpatient studies in a university setting or outpatient clinics. The 23 studies consisted of n=2,085 participants, with 74% being female, ages ranging from 12 – 62 years and study duration from two weeks to two years. All participants were overweight or obese at baseline. We found that in 17 of 23 eligible studies, a significant increase in 25OHD was observed with a decrease in weight (Christensen et al., 2012; Damms-Machado et al., 2012; Gangloff et al., 2015; Holecki et al., 2007; Holecki et al., 2008; Ibero-Baraibar et al., 2015; Josse et al., 2011; Lucey et al., 2008; Mason et al., 2011a; Ortega et al., 2009; Reinehr et al., 2007; Riedt et al., 2007; Shahar et al., 2010; Tzotzas et al., 2010; Van Loan et al., 2011; Wamberg et al., 2013; Zittermann et al., 2009), with a decrease in BMI in 15 studies (Albadah et al., 2015; Christensen et al., 2012; Gangloff et al., 2015; Holecki et al., 2007; Holecki et al., 2008; Ibero-Baraibar et al., 2015; Josse et al., 2011; Lucey et al., 2008; Mason et al., 2011b; Ortega et al., 2009; Reinehr et al., 2007; Tzotzas et al., 2010; Van Loan et al., 2011; Wamberg et al., 2013; Zittermann et al., 2009), with a

decrease in %FM in 14 studies (Apovian et al., 2009; Christensen et al., 2012; Damms-Machado et al., 2012; Gangloff et al., 2015; Holecki et al., 2008; Ibero-Baraibar et al., 2015; Josse et al., 2011; Mason et al., 2011a; Ortega et al., 2009; Reinehr et al., 2007; Riedt et al., 2007; Tzotzas et al., 2010; Van Loan et al., 2011; Zittermann et al., 2009) and with a decrease in FFM in four studies (Christensen et al., 2012; Josse et al., 2011; Ortega et al., 2009; Riedt et al., 2007) (Table 4.1). Overall, five of these studies reported a significant association ($p<0.05$) between 25OHD and any index of weight change (Christensen et al., 2012; Damms-Machado et al., 2012; Gangloff et al., 2015; Reinehr et al., 2007; Wamberg et al., 2013) and one study reported a non-significant association (Tzotzas et al., 2010) (Table 4.1).

Nine of 23 trials were assessed as high quality studies (Jadad ≥3) and consisted of n=1104 participants, with 80% being female, ages ranging from 32 – 58 years (Chan She Ping-Delfos, 2009; Cummings, 2006; Ibero-Baraibar et al., 2015; Lucey et al., 2008; Mason et al., 2011a; Ricci et al., 1998; Riedt et al., 2007; Shahar et al., 2010; Zittermann et al., 2009). Of these nine studies, a significant increase in 25OHD was observed with a decrease in weight in five studies (Ibero-Baraibar et al., 2015; Mason et al., 2011a; Riedt et al., 2007; Shahar et al., 2010; Zittermann et al., 2009), a decrease in BMI in three studies (Ibero-Baraibar et al., 2015; Mason et al., 2011a; Zittermann et al., 2009), a decrease in %FM in four studies (Ibero-Baraibar et al., 2015; Mason et al., 2011a; Riedt et al., 2007; Zittermann et al., 2009), and a decrease in FFM in one study (Riedt et al., 2007).

Table 4.1 Human trials on weight loss and change in vitamin D status.

First author and year of publication	Study details	Jadad score	Weight loss strategy	Vitamin D in food (IU/day)	Increase in vitamin D status (nmol/L)	Decrease in weight (kg)	Decrease in BMI (kg/m^2)	Decrease in FM (%)	Decrease in FFM (kg)	Assay
Ricci et al. (1998)	Age: 60 y Subjects: n=30 (F) Duration: 24 w Location: USA Study type: RCT	4	CR	NR	No change	Yes	Yes	Yes	Yes	RIA
Jensen et al. (2001)	Age: NR Subjects: n=52 (F) Duration: 24 w Location: Denmark Study type: RCT	0	CR	CR: 200 Control: 200	No change	Yes	Yes	NR	NR	CBA
Cummings (2006)	Age: 53 y Subjects: n=29 (6 M, 23 F) Duration: 12 w Location: Australia Study type: RCT	3	CR	CR: 78 Control: 78	No change	Yes	Yes	Yes	Yes	RIA

Holecki et al. (2007)	Age: 50 y Subjects: n=62 (F) Duration: 12 w Location: Poland Study type: Single stranded study	0	CR & PA	NR	Yes	Yes	Yes	NR	NR	RIA
Reinehr et al. (2007)	Age: 12 y Subjects: n=156 (79 M, 77 F) Duration: 1 y Location: Germany Study type: Single stranded study	0	CR & PA	CR & PA: 39	Yes	Yes, and significantly associated (r= -0.27, p=0.013)	Yes	Yes	NR	CLIA
Riedt et al. (2007)	Age: 38 y Subjects: n=31 (F) Duration: 24 w Location: USA Study type: RCT	4	CR	NR	No change	Yes	NR	Yes	Yes	RIA
Holecki et al. (2008)	Age: 49 y Subjects: n=20 (F) Duration: 12 w Location: Poland Study type: RCT	0	CR & PA	CR & PA: NR	No change	Yes	Yes	Yes	NR	RIA

Lucey et al. (2008)	Age: 32 y Subjects: n=276 (118 M, 158 F) Duration: 8 w Location: Iceland, Spain, Ireland Study type: RCT	3	CR	CR 1: 68 CR 2: 56 CR 3: 420 Control: 64	Yes (1 group) Decrease (3 groups)	Yes	Yes	NR	NR	ELISA
Apovian et al. (2009)	Age: NR Subjects: n=40 (12 M, 28 F) Duration: 12 w Location: USA Study type: Single stranded study	0	CR	NR	Yes	No change	No change	Yes	NR	NR
Ortega et al. (2009)	Age: 27 y Subjects: n=61 (F) Duration: 2 w Location: Spain Study type: RCT	1	CR	CR 1: 128 CR 2: 260	Yes	Yes	Yes	Yes	Yes	RIA
Chan She Ping-Delfos (2009)	Age: 57 y Subjects: n=43 (23 M, 20 F) Duration: 12 w Location: Australia Study type: RCT	3	CR, CR & PA	CR 1: 83 CR 2: 129 CR 3 & PA: 131	No change	Yes	Yes	Yes	Yes	RIA

Zittermann et al. (2009)	Age: 48 y Subjects: n=83 (22 M, 61 F) Duration: 1 y Location: Germany Study type: RCT	5	CR	CR: 80	Yes	Yes	Yes	Yes	NR	RIA
Shahar et al. (2010)	Age: 52 y Subjects: n=126 (M, F) Duration: 2 y Location: Israel Study type: RCT	3	CR	NR	Yes	Yes	NR	NR	NR	CLIA
Tzotzas et al. (2010)	Age: 40 y Subjects: n=62 (F) Duration: 20 w Location: Greece Study type: Single stranded study	0	CR	NR	Yes	Yes, and associated (r= -0.367, p=0.065)	Yes, and associated (r= -0.376, p=0.059)	Yes	NR	ECLIA

Josse et al. (2011)	Age: 28 y Subjects: n=81 (F) Duration: 16 w Location: Canada Study type: RCT	2	CR & PA	CR & PA 1: 28 CR & PA 2: 392 CR & PA 3: 528	Yes (1 group) No change (1 group) Decrease (1 group)	Yes	Yes	Yes	Yes (2 groups) Decrease (1 group)	RIA
Mason et al. (2011a)	Age: 58 y Subjects: n=439 (F) Duration: 1 y Location: USA Study type: RCT	3	CR, CR & PA, PA	CR: 540 CR & PA: 538 PA: 595 Control: 447	Yes**	Yes	Yes	Yes	No	CLIA
Van Loan et al. (2011)	Age: 32 y Subjects: n=71 (21 M, 50 F) Duration: 12 w Location: USA Study type: RCT	0	CR	CR 1: 128 CR 2: 320	Yes (1 group) No change (1 group)	Yes	Yes	Yes	No change	RIA
Christensen et al. (2012)	Age: 62 y Subjects: n=175 (33 M, 142 F) Duration: 16 w Location: Denmark Study type: Single stranded study	0	CR	CR: 382	Yes*	Yes, and significantly associated (r= -0.21, p=0.006)	Yes	Yes, and significantly associated (r= -0.16, p=0.03)	Yes	CLIA

Damms-Machado et al. (2012)	Age: 47 y Subjects: n=32 (4 M, 28 F) Duration: 12 w Location: Germany Study type: Single stranded study	0	CR	CR: 200	Yes*	Yes	NR	Yes, and significantly associated (r= -0.6369 p<0.0001)	NR	RIA
Wamberg et al. (2013)	Age: 35 y Subjects: n=17 (9 M, 8 F) Duration: 8 w & 4 w maintenance Location: Denmark Study type: Single stranded study	0	CR	NR	Yes	Yes, and significantly associated (% weight loss) (r= 0.67, p=0.005)	Yes, and significantly associated (r= -0.67, p=0.005)	NR	NR	LC-MS
Albadah et al. (2015)	Age: 32 y Subjects: n=49 (M) Duration: 12 w Location: Saudi Arabia Study type: Single stranded study	0	CR	NR	Yes	NR	Yes	NR	NR	NR
Ibero-Baraibar et al. (2015)	Age: 57 y Subjects: n=47 (24 M, 23 F) Duration: 4 w	5	CR	CR 1: 142 CR 2: 191	Yes**	Yes	Yes	Yes	NR	RIA

	Location: Spain Study type: RCT									
Gangloff et al. (2015)	Age: 48 y Subjects: n=103 (M) Duration: 1 y Location: Canada Study type: Single stranded study	0	CR & PA	NR	Yes*	Yes, and significantly associated (r= -0.31, p<0.005)	Yes, and significantly associated (r= -0.32, p<0.005)	Yes, and significantly associated (r= -0.32, p<0.005)	NR	LC-MS

Legend: Age, mean age; CBA, Competitive protein binding assays using rachitic rat kidney cytosol; CLIA, Chemiluminescence immune assays; CR, Caloric restriction; ECLIA, Electrochemiluminescence immunoassay; ELISA, Enzyme-linked immune absorbent assay; F, Female; LC-MS, Liquid chromatography mass spectrometry; M, Male; NR, Not reported; PA, Physical activity; RCT, Randomiced controlled trial; RIA, Radioimmunoassay; w, Weeks; y, Year/s; *, Baseline 25OHD <50 nmol/L, after weight loss were >50 nmol/L; **, Baseline 25OHD <50 nmol/L, after weight loss were >50 nmol/L (1 study arm only); $p<0.05$ was considered significant.

4.3.2 Meta-regression analysis

Meta-regression models were run unadjusted and adjusted for the effect of the confounders. However none of the confounders was found to make a significant contribution to the change in the WMD of vitamin D when tested individually or in combination. We hence report the results obtained from the unadjusted regression analyses on 1) all weight loss studies (Table 4.2) and 2) adjusted for study quality (Table 4.3).

4.3.2.1 The effect of weight loss

The meta-regression analysis for 34 arms of 17 weight loss studies included 1,522 subjects and a mean age of 45 years (Chan She Ping-Delfos, 2009; Cummings, 2006; Damms-Machado et al., 2012; Gangloff et al., 2015; Holecki et al., 2007; Holecki et al., 2008; Ibero-Baraibar et al., 2015; Jensen et al., 2001; Josse et al., 2011; Lucey et al., 2008; Mason et al., 2011a; Ortega et al., 2009; Ricci et al., 1998; Riedt et al., 2007; Tzotzas et al., 2010; Van Loan et al., 2011; Zittermann et al., 2009). The relationship favoured a marginally significant increase of 6.0 nmol/L (95% CI: -12.42, +0.47) in the WMD of 25OHD for an average weight loss of 10 kg (p=0.06) (Table 4.2). When adjusting for quality of study this association was close to significance (p=0.05), with an increase of 6.4 nmol/L (95% CI: -12.85, +0.12) in WMD of 25OHD for weight loss of 10 kg (Table 4.3).

4.3.2.2 The effect of change in %FM

The meta-regression analysis for 28 arms of 13 weight loss studies included 1,346 subjects, with a mean age of 44 years (Chan She Ping-Delfos, 2009; Cummings, 2006; Gangloff et al., 2015; Holecki et al., 2008; Ibero-Baraibar et al., 2015; Josse et al., 2011; Lucey et al., 2008; Mason et al., 2011a; Ortega et al., 2009; Riedt et al., 2007; Tzotzas et al., 2010; Van Loan et al., 2011; Zittermann et al., 2009). Results were marginally significant with an increase of 9.1 nmol/L (95% CI: -19.69, +1.57) in the WMD of 25OHD for a 10% loss in

%FM (p=0.08) (Table 4.2). This result approached significance when analysis was adjusted for quality of studies where an increase of 10.5 nmol/L (95% CI: -21.87, +0.85) in the WMD of 25OHD for a 10% decrease in %FM (p=0.06) was observed (Table 4.3).

4.3.2.3 The effect of change in BMI, and FFM

The meta-regression analyses failed to find any significant relationship between the change in BMI and in FFM and the WMD of vitamin D in all study arms even when adjusted for quality of study (Table 4.2 & 4.3).

Table 4.2 Unadjusted meta-regression of changes in weight and indices of body composition on vitamin D status.

Outcome variable: WMD of vitamin D status	Variable	Estimated coefficient β	95% CI	p value
The effect of weight loss				
Model 1	Change in weight (kg)	-0.60	-1.24, 0.04	0.06
	Constant	-0.30	-4.24, 4.79	0.90
The effect of decrease in BMI				
Model 2	Change in BMI (kg/m^2)	-0.13	-4.67, 4.41	0.95
	Constant	2.40	-6.98, 11.78	0.60
The effect of %FM loss				
Model 3	Change in FM (%)	-0.91	-1.96, 0.15	0.08
	Constant	2.34	-1.22, 5.89	0.18
The effect of FFM loss				
Model 4	Change in FFM (kg)	1.28	-1.25, 3.81	0.30
	Constant	4.68	1.29, 8.07	0.01

Legend: BMI, body mass index; FFM, fat free mass; %FM, percentage fat mass; WMD, weighted mean difference (nmol/L) in vitamin D status; $p<0.05$ was considered significant.

Table 4.3 Meta-regression of changes in weight and indices of body composition on vitamin D status adjusted for study quality.

Outcome variable: WMD of vitamin D status	Variable	Estimated coefficient β	95% CI	p value
The effect of weight loss				
Model 1	Change in weight (kg)	-0.64	-1.28, 0.01	0.05
	Jadad score (0, 1)	1.57	-2.71, 5.86	0.46
	Constant	-0.84	-6.22, 4.55	0.75
The effect of decrease in BMI				
Model 2	Change in BMI (kg/m^2)	-0.17	-4.77, 4.42	0.93
	Jadad score (0, 1)	0.82	-4.82, 6.46	0.76
	Constant	1.88	-8.28, 12.04	0.70
The effect of %FM loss				
Model 3	Change in FM (%)	-1.05	-2.18, 0.08	0.06
	Jadad score (0, 1)	1.43	-2.45, 5.31	0.45
	Constant	1.04	-3.97, 6.05	0.67
The effect of FFM loss				
Model 4	Change in FFM (kg)	2.11	-0.55, 4.77	0.11
	Jadad score (0, 1)	5.30	-1.35, 11.96	0.11
	Constant	1.07	-4.56, 6.69	0.69

Legend: BMI, body mass index; FFM, fat free mass; %FM, percentage fat mass; Jadad score (0=0 <3;1=1 ≥3); WMD, weighted mean difference (nmol/L) in vitamin D status; $p<0.05$ was considered significant.

4.4 Discussion

It is yet to be confirmed whether vitamin D is sequestered or undergoes a volumetric dilution in obesity. We questioned whether weight loss in the absence of vitamin D supplementation, would increase circulating 25OHD. In this systematic review 17 out of 23 studies observed an increase in 25OHD with weight loss, but only five of these studies reported a significant correlation coefficient between the two variables (Christensen et al., 2012;

Damms-Machado et al., 2012; Gangloff et al., 2015; Reinehr et al., 2007; Wamberg et al., 2013). Our meta-regression analysis indicated a near significant association between weight loss and increase 25OHD, which suggested that for every 10 kg mean weight loss there could be an average increase of 6.0 nmol/L in the WMD of 25OHD (Table 4.2, Figure 4.3). When we adjusted the analysis for quality of study (with a Jadad score of ≥3 indicative of high quality), the relationship between change in weight and 25OHD was still near significance with no major change in the regression slope (Table 4.3). Hence it would appear that body weight does contribute to a volumetric dilution of 25OHD (Drincic et al., 2012).

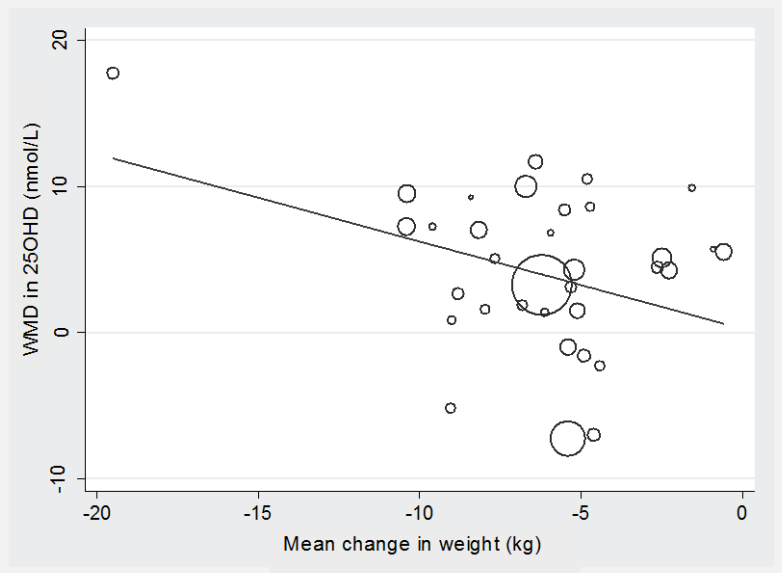

Figure 4.3 Relationship between change in weight (kg) and change in vitamin D status (WMD). Bubble plot of fitted metaregression line; the size of the bubbles is proportional to the precision of the estimate; WMD, weighted mean difference (nanomoles per liter) in vitamin D status.

Following caloric restriction, FM loss is a major portion of weight loss (Varady, 2011). While FFM is also lost, the precise amount could vary with protein intake and/or increased

physical activity (Stiegler & Cunliffe, 2006), with both generally retarding loss of FFM. As both FM and FFM are major stores for the vitamin (Heaney et al., 2009), we examined the effect of changes in these compartments on circulating 25OHD.

Fourteen of 23 studies observed that with a loss in %FM there was an increase in 25OHD (Apovian et al., 2009; Christensen et al., 2012; Damms-Machado et al., 2012; Gangloff et al., 2015; Holecki et al., 2008; Ibero-Baraibar et al., 2015; Josse et al., 2011; Mason et al., 2011a; Ortega et al., 2009; Reinehr et al., 2007; Riedt et al., 2007; Tzotzas et al., 2010; Van Loan et al., 2011; Zittermann et al., 2009) with three of these studies indicating a significant correlation coefficient between the two variables (Christensen et al., 2012; Damms-Machado et al., 2012; Gangloff et al., 2015). Meta-regression analysis found that decreases in %FM were not significantly related to increases in 25OHD, on examination of the total dataset (Table 4.2). When adjusted for quality of trials this relationship between %FM loss and increase in 25OHD became marginally significant (Table 4.3, Figure 4.4). The lack of statistical significance may have arisen from the smaller number of studies reporting %FM (28 study arms) and the smaller sample size (n=1,346) but also from the larger spread of effects. The β coefficient suggested that with a 10% loss in %FM, the mean increase in 25OHD would be 10.5 nmol/L, however the 95% CI were large at 21.8, -0.8 nmol/L. The amount of vitamin D available in adipose tissue is approximately 103 nmol/kg (Heaney et al., 2009), and represents a sizeable store in an obese person. Based on this, a 10% decrease in %FM should have resulted in a much greater increase in 25OHD than predicted by the β coefficient. There are a few potential reasons that may explain our observations.

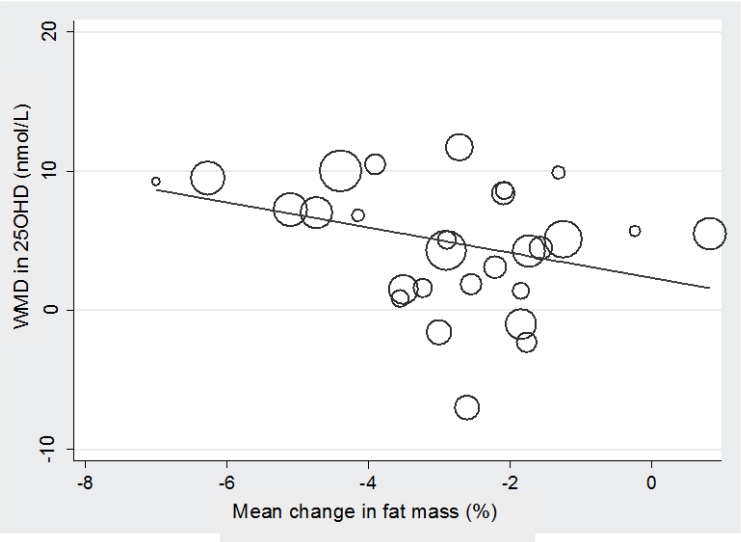

Figure 4.4 Relationship between change in FM (percentages) and change in vitamin D status (WMD). Bubble plot of fitted metaregression line; the size of the bubbles is proportional to the precision of the estimate; WMD, weighted mean difference (nanomoles per liter) in vitamin D status.

The detection of changes in 25OHD and %FM loss would be influenced by the sensitivity of the various methods used in these trials. There is substantial inter-laboratory variation in detecting 25OHD (Binkley et al., 2004). Binkley et al. (2004) observed that there could be up to a two-fold difference between laboratories assaying the same sample with the same technique. Similarly body composition techniques have limitations in their calibration, accuracy and precision (Erselcan et al., 2000) dependent on the type of technique used (BIA vs. DEXA vs. skin fold measurement) (Erselcan et al., 2000; Neovius, Hemmingsson, Freyschuss, & Udden, 2006) or within models of the same machine (Pearson, Horton, & Green, 2011). DEXA is considered to be the gold standard for body composition, however variations up to 6% have been found between instruments from the same manufacturer (Covey, Berry, & Hacker, 2010; Ellis & Shypailo, 1998). Moreover BIA and the skinfold technique appear to be more accurate in non-obese subjects, so there could be substantial variation in the studies of obese subjects included in this review (Apovian et al., 2009; Damms-Machado et

al., 2012; Holecki et al., 2008; Lucey et al., 2008; Ortega et al., 2009; Reinehr et al., 2007; Tzotzas et al., 2010; Zittermann et al., 2009). Overall, it is possible that changes in 25OHD due to fat loss, and changes in %FM may not have been appropriately detected.

A second reason for the small increase in 25OHD on %FM loss is that once taken up into adipose tissue, 25OHD is released very slowly back into circulation. The latter may protect the individual from large sudden increases of a potentially toxic nutrient, while acting as a store in times of need (Rosenstreich et al., 1971). Another further possibility is a negative feedback loop where higher circulating 1,25(OH)D in obesity disables the production of 25OHD (Bell et al., 1985; Pourshahidi, 2015). The mechanism that controls this slow release is uncertain, however if this is true then the shorter duration studies in this review may not detect this return of 25OHD. Importantly, vitamin D is involved in both paracrine and autocrine actions in adipose tissue (Morris & Anderson, 2010). A nVDR is expressed in adipose tissue (Salehi-Tabar et al., 2012), and most tissues that express VDR also contain the enzyme CYP27B1 for conversion of the circulating metabolite, 25OHD to $1,25(OH)_2D_3$. Adipose tissue has the ability to synthesise and degrade vitamin D for autocrine and paracrine use, such as in adipogenesis, lipid metabolism and inflammation in obesity (Calton, Keane, & Soares, 2015b; Ding et al., 2012). The expanded adipose tissue in obesity may hence engender an increased requirement of 25OHD, resulting in less return to the circulation following weight loss.

We now know that there is extensive metabolic conversion of vitamin D in storage depots. Adipose tissue is a dynamic endocrine organ, containing a variety of hydroxylase enzymes. These include the hydroxylase to convert cholecalciferol to 25OHD to $1,25(OH)_2D_3$, as well as the catabolic 24-hydroxylase for degradation of calcitrol to calcitroic acid, and 25OHD to $24,25(OH)_2D_3$ to 1-desoxycalcitroic acid, the major metabolite of 25OHD (Ding et al., 2012; Jones, Prosser, & Kaufmann, 2012; Wamberg et al., 2013). Calcitroic acid and 1-desoxycalcitroic acid are excreted through the bile into faeces (Jones, Strugnell, & DeLuca, 1998a), with limited amounts found in the urine (Kumar, Harnden, & DeLuca, 1976). These

metabolites are eliminated from the system, and would not be detected in studies that only sampled the plasma compartment. In addition, there is emerging data to indicate that enzyme expression also varies with fat depots (subcutaneous vs. visceral) and degrees of fatness (lean vs. obese) (Wamberg et al., 2013). Our current understanding is that lean and obese have similar expression of the enzyme CYP27A1 that converts 25OHD to $1,25(OH)_2D_3$, and similar expression of CYP24A1 that degrades 25OHD to calcitroic acid (Jones et al., 2012). However in response to weight loss, obese subjects show an elevation of the catabolic 24-hydroxylating enzyme, CYP2J2 (Wamberg et al., 2013), which results in several (almost 30) inactive metabolites (Ding et al., 2012; Jones et al., 2012). Some of these include $1,24,25(OH)_3D_3$, 24-oxo and/or 23-hydroxy groups (Jones, Strugnell, & DeLuca, 1998b) which represent pathways for vitamin D elimination (Ross et al., 2011). Overall, many of these factors would be operative at the same time during weight loss and would go some way in explaining the smaller increase in 25OHD observed in this review. Perhaps, as Rosenstreich et al. (1971) opined, this slow return of 25OHD acts to protect against vitamin D toxicity that may occur if a flood of the nutrient became available during weight loss.

There are now many studies that show a positive relationship between 25OHD and muscle mass, growth, and strength/function (Girgis, Clifton-Bligh, Hamrick, Holick, & Gunton, 2013; Pojednic & Ceglia, 2014; Sato, Iwamoto, Kanoko, & Satoh, 2005). This would suggest that higher vitamin D status may improve muscle mass and function, relative to those who are vitamin D insufficient. As FFM is a major store for the nutrient, we assumed that during weight loss, mobilisation of the protein mass would contribute to an increase in 25OHD. However, we did not obtain a significant relationship between FFM loss and vitamin D status (Table 4.3). In fact, the slope of the regression line was in the opposite direction to that hypothesized (Table 4.3, Figure 4.5). Since the majority of studies reviewed showed a small to moderate decrease in FFM, our interpretation would be that a loss in FFM decreased circulating 25OHD. We acknowledge that this was a non-significant outcome but the slope was similar or greater than that obtained with %FM (Table 4.3, Figure 4.4). Could this observation suggest that the

expected positive relation between vitamin D status and muscle mass (Girgis et al., 2013) is possibly bi-directional? Such a phenomenon could have negated the rise in 25OHD seen with loss of %FM (Figure 4.4), and perhaps explain why the slope of weight loss change predicted was lesser than that obtained with %FM (Table 4.3). It is unclear why a loss in FFM should decrease 25OHD. Fat free mass is comprised of lean tissue (mainly skeletal muscle) and bone mineral content. As bone mass is also lost during caloric restriction, vitamin D may prevent the expected decrease in fractional bone mass that occurs with weight loss (Shapses & Sukumar, 2012). There is good cross talk between muscle and bone, and adipose tissue and bone (Shapses & Sukumar, 2012). So a decrease in 25OHD, or a less than expected increase, may also indicate diversion from plasma to other tissues during weight loss.

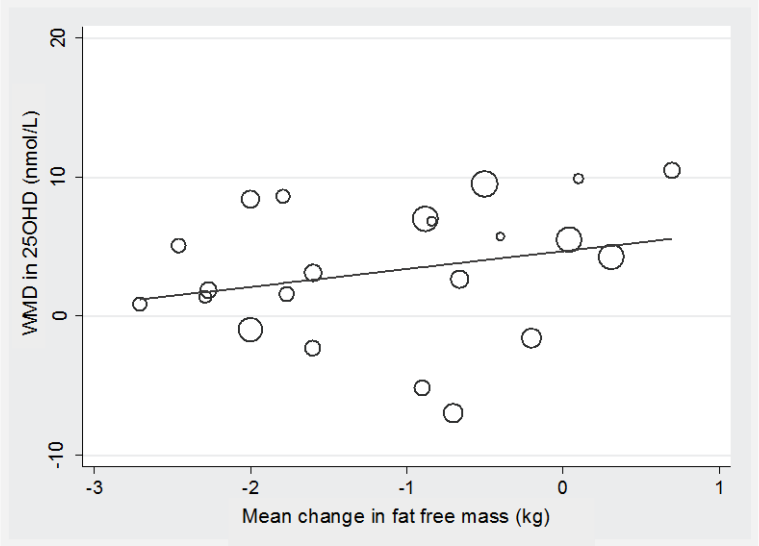

Figure 4.5 Relationship between change in FFM (kilograms) and change in vitamin D status (WMD). Bubble plot of fitted metaregression line; the size of the bubbles is proportional to the precision of the estimate; WMD, weighted mean difference (nanomoles per liter) in vitamin D status.

4.5 Limitations

To the best of our knowledge, there are no other systematic reviews that have specifically addressed our question. Hence we are unable to compare our results to the existing literature. Our search of the literature was over a long interval but covered only two prominent databases.

We identified 23 studies, of which only nine were quality studies with a sample size of n=1,104. Hence our meta-regression analysis may be limited by adequate numbers, despite contacting the authors of all studies for additional information. Seasonality of 25OHD can amount to 17 nmol/L (Daly et al., 2012; van der Wielen et al., 1995) and have a major confounding on the stated outcomes. We were unable to control for this confounder in our analysis since season of start and completion was only mentioned in three studies. Moreover there was a range of assays used in these studies as well as a range of body composition techniques that would have influenced our outcomes.

4.6 Future research

High quality trials (Jadad ≥3) of at least six months duration, that target a substantial amount of weight loss (~20% fat loss) are required. Such data would cement the clinical relevance of weight loss in normalising vitamin D status of the obese individual. Adipose tissue is an active endocrine organ, expressing numerous receptors including vitamin D (Ding et al., 2012; Kershaw & Flier, 2004), and vitamin D is required for normal formation and function of adipose tissue (Kamei et al., 1993; Kong & Li, 2006; Li et al., 2008; Wong et al., 2011). Hence what happens to vitamin D status before and after weight loss is important. Further investigation into vitamin D concentration in adipose tissue of obese subjects, pre and post-weight loss may provide an insight into the amount of vitamin D and its metabolites at a cellular level. Wamberg et al. (2013) have provided some pioneering data in the area of vitamin D hydroxylation and catabolizing enzymes in adipose tissue. These data need validation in future trials as they provide an understanding of the dynamic influence of adipose tissue on vitamin D metabolism. Furthermore trials on vitamin D need to report detailed body compositional changes, including body fat distribution. DEXA is now globally available and changes in android:gynoid fat can be reported, though more sophisticated determinations of subcutaneous and visceral adipose tissue changes based on CT or MRI scans would be worthwhile inclusions. Lastly, there is a need to report the concentrations of inactive

compounds of vitamin D metabolism in both urine and serum. This would assist the evaluation of how much of the vitamin is unavailable for metabolism during weight loss.

4.7 Conclusion

This systematic review provides good evidence for an inverse relationship between weight and fat loss and 25OHD in obesity. While overall in support of a volumetric dilution phenomenon in obesity, the review cannot discount a sequestration effect and possibly, extensive degradation of 25OHD after weight loss.

4.8 Acknowledgements

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4.9 Author contribution

PKP conducted the literature search, assembled the tables, extracted the data and co-wrote the manuscript. MJS generated the idea, conceptualized the review process, cross-checked data extraction and co-wrote the manuscript. YZ prepared the data extraction template, conducted the statistical analysis, and co-wrote the manuscript. All authors contributed to the writing of the final manuscript.

Chapter 5 The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor Survey

The content of this chapter is covered by a published paper (Appendix C).
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Pannu, P. K., Zhao, Y., Soares, M. J., Piers, L. S., & Ansari, Z. (2016). The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor survey. *Public Health Nutrition*, 1-12. doi:10.1017/S1368980016001609

Objective addressed

Objective 4a: To investigate the association between vitamin D status and dietary calcium intake and the presence of MetS.

5.1 Introduction

Vitamin D is a secosteroid that is cutaneously produced through solar UVB irradiation of 7-dehydrocholesterol present in the skin (Liu, 2012; Pilz et al., 2013). The second source of vitamin D is via food intake, and as for calcium, the greatest contribution comes from milk and other dairy product intake. Vitamin D undergoes two hydroxylation steps one in the liver and one in the kidney. The final hydroxylation step in the kidney converts 25OHD to its active metabolite, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), and the enzyme 1-alpha hydroxylase catalyzes this conversion (Wamberg et al., 2013). Interestingly the expression of the nVDR and 1-alpha hydroxylase is present not only in the kidneys, but many other tissues of the body (Dusso, Brown, & Slatopolsky, 2005) including the pancreas (Pilz et al., 2013) and immune cells (Bouillon et al., 2014; Calton et al., 2015b; Hossein-Nezhad et al., 2013; Neve et al., 2013). Thus many tissues have the ability to locally synthesize $1,25(OH)_2D$ from 25OHD and potentially contribute to circulating concentrations (Dusso et al., 2005). The active metabolite can then bind to the nVDR, where it forms a heterodimer with the retinoid X receptor (Pilz et al., 2013). It is now recognised that nVDR regulates approximately 3% of the human genome (~700 genes) (Bouillon et al., 2014; Pilz et al., 2013) and together with its wide distribution, provides some foundation for the study of extra-skeletal benefits of vitamin D.

The MetS is a clustering of risk factors that greatly increases the risk of CVD and diabetes T2DM. Insulin resistance is a key player in the development of MetS, however factors other than IR are also important. Clinical diagnosis of MetS is based on the presence of three or more of the following markers of chronic disease: greater WC, raised FPG, hypertension (SBP) or DBP), and dyslipidaemia (raised TG and low HDL-C) (Alberti et al., 2009). The prevalence of MetS in Australia is high with ~30% of adults classified as having the syndrome (Cameron et al., 2007); a figure that is comparable to other developed countries (Beltran-Sanchez, Harhay, Harhay, & McElligott, 2013; Grundy, 2008). For a sun-drenched country

with abundant milk supplies, it is surprising that both vitamin D insufficiency and low calcium intake are highly prevalent in Australia (Australian Bureau of Statistics, 2014; Daly et al., 2012; Nowson et al., 2012). Inadequate vitamin D status has been implicated as a causal factor in many chronic conditions (Peterlik, Boonen, Cross, & Lamberg-Allardt, 2009) including T2DM (Brock et al., 2011), MetS (Brenner et al., 2011; Gagnon et al., 2012), CVD (Anderson et al., 2010), hypertension (Kunutsor et al., 2013; Scragg, Sowers, & Bell, 2007) and IR (Chiu et al., 2004; von Hurst et al., 2010). We have previously discussed the potential for calcium and vitamin D to regulate body weight (Zemel et al., 2000) and influence the risk of chronic disease (Soares et al., 2011a; Soares et al., 2011b; Soares et al., 2012; Soares, Pathak, & Calton, 2014). Documented pathways include calcium's stimulation of fat oxidation, heightened diet induced thermogenesis, increased faecal fat excretion (Soares et al., 2012), reduced circulating TG (Major et al., 2007) and the potential for vitamin D to increase resting metabolism (Calton et al., 2015c). Emerging data also support a beneficial effect on IR and T2DM (Davies et al., 2000; Drolet et al., 2008; Pittas et al., 2006; Soares et al., 2012). However, a consensus document produced by the IOM found little convincing evidence available then, in support of extra-skeletal effects of vitamin D (Institute of Medicine, 2011). The aim of this study was to investigate whether there was a link between population based measures of vitamin D status, dietary calcium intake, and the prevalence of MetS.

5.2 Methods

To fulfil our objectives we used a state-wide representative survey of Victorian adults; The VHM (Department of Health, 2012a). The VHM was conducted between May 2009 and April 2010. A stratified cluster sample was selected, based on Census Collection Districts (CDs) within the eight Victorian Government Department of Health regions. Fifty randomly selected CDs were included in the sample, 25 from metropolitan and 25 from rural Victoria. One eligible person (aged 18–75 years) from each household, in each CD was randomly selected to participate. The VHM was approved by the Human Research Ethics Committee (HREC) of the Baker IDI Heart and Diabetes Institute, Melbourne, Victoria (Kelsall, de

Gooyer, Carey, Vaughan, & Ansari, 2013). The analysis of the VHM database was also approved by the HREC at Curtin University (HREC approval number: SPH-19-2014).

The VHM involved an initial household visit to participants, to collect demographic information, followed by a visit to a local test site to collect risk factor information and undergo biomedical and physical examination. Participants were then asked to complete three 24 hour dietary recall interviews, which were conducted over a six-week period. The overall response rate for the VHM was 38% and a final sample of 3,653 participants was achieved.

The response rate in the VHM survey is comparable to similar Australian surveys including the Australian Health Survey: Biomedical Results 2011-12 (response rate 37.1%) (Australian Bureau of Statistics, 2013a), and the AusDiab study (response rate 37%) (Dunstan et al., 2002). To identify any potential selection bias in the VHM between participants and non-participants, key demographic characteristics were compared. A minimal level of difference was found between the two groups (Kelsall et al., 2013). Demographic characteristics of participants of the VHM survey were also similar to those from the annual Victorian Population Health Surveys (VPHS) conducted in 2010 (n=7,535) and 2011-12 (n=33,673), by the Victorian Government, which had response rates of 73% (Department of Health, 2012c) and 67% (Department of Health, 2014) respectively. This would suggest that the level of bias in the VHM is probably no different from the larger VPHS.

Test sites for the collection of biomedical and physical measures were set-up specifically for the purposes of the study in CDs included in the sample. The procedures used for the biomedical examination were closely aligned with the protocol recommended by the WHO (World Health Organization, 1999). Participants provided written informed consent upon arrival at test sites and were asked to stay until all tests were complete. Abnormal test results were reviewed by a study doctor who determined whether a result warranted follow-up with a participant. Further details on the survey protocols and procedures can be found in the VHM

report (Department of Health, 2012a), and the VHM Food and Nutrition report (Department of Health, 2012b).

5.2.1 Sample

In this study, we excluded participants with: missing HbA1c data (n=31), those with HbA1c ≥6.7% as they were classified as having T2DM according to the American Diabetes Association cut-offs (n=39) (American Diabetes Association, 2010), those with diagnosed T2DM (n=140), those with type one diabetes (n=9), those on diabetic medications (n=25), and those with missing metabolic components for MetS diagnosis (n=5). Hence, a total of 3,404 subjects were included in this analysis. Information on the use of supplements (calcium or vitamin D) was not available in this survey.

5.2.2 Assessment of vitamin D status

Blood samples were collected via venepuncture after an overnight fast of ten hours or more. Blood was immediately transported to an accredited central laboratory in Melbourne, Australia. The measurement of serum 25OHD concentration was based on DiaSorin Corporation Liaison® 25OHD total assay. The assay is an automated direct competitive chemiluminescent immunoassay that measures D_2 and D_3 to provide a total value for circulating vitamin D in nmol/L. The detection limit was 10 nmol/L. The All Laboratory Trimmed Mean (ALTM) was not computed by the laboratory, nor were results compared to a 'Target Value' (TV) assigned by the NIST Reference Measurement Procedure.

5.2.3 Assessment of dietary calcium intake

Dietary intake data was obtained by multiple-pass 24-hour diet recall using computer assisted telephone interviews (CATI). The first diet recall interview was conducted within five to seven days of the participants attending the biomedical examination. Two subsequent diet recall interviews were conducted at two-week intervals following the first diet recall interview. A total of 3,653 participants attended and participated in survey components at test sites. Three

dietary recalls were conducted, with a total of 10,307 dietary recalls completed, where: 96% completed one dietary recall, 94% completed two dietary recalls, and 92% completed three dietary recalls. Details of the dietary recall and post interview processing methodology employed have been described in detail in the VHM report (Department of Health, 2012b).

All dietary recall interviews were conducted by certified dietitians from the Department of Nutrition and Dietetics, Monash University. Interviewers were trained to assure competency and consistency in collected dietary recall information. Interviewers used a food model book to aid participants with their description of portion sizes of the food and beverages they had consumed. The food model book prompted dietary recall by including frequently forgotten foods and eating occasions, and assisted with portion size estimation with 'to scale' photos of food and beverage containers, measuring spoons and cups (Department of Health, 2012b).

The FoodWorks® nutrition software (FoodWorks® software, FoodWorks Interview) were employed for implementation of dietary recalls. The dietary recall used a multiple-pass approach to assist participants to sufficiently recall their food and beverage intake. The software includes a scripted guide for interviewers to help prompt participants for food recall in each interview. Interviewers were able to interrupt and prompt for further details on food items if required. Further information on the multiple-pass dietary recall process have been described in detail in the VHM report (Department of Health, 2012b).

On completion of the interviews, volume conversion factors were developed to convert food volumes into food weights. Conversion of food volume to weights were done by "reference to published data, by measuring the weight and volume of specific foods, or by considering the food as very similar to another food for which a volume conversion factor was already available" (Department of Health, 2012b). The AUSNUT 2007 (Food Standards Australia New Zealand, 2007) nutrient composition data were referred to calculate nutrient intakes based on estimated food intake. The mean intake for each nutrient was computed for each participant based on information collected from three 24 hour dietary recalls and was

used in the analysis. This information was used to get a single measure of nutrient intake for each participant (Department of Health, 2012b).

5.2.4 Physical activity level

The following criteria were used to define each participant's level of physical activity: sufficiently physically active (≥150 minutes of 'physical activity time' per week); insufficiently physically active (1-149 minutes of 'physical activity time' per week); and physically inactive (0 minutes of 'physical activity time' per week) (Department of Health, 2012a). 'Physical activity time' was calculated as the sum of the time spent walking or performing moderate activity plus double the time spent in vigorous physical activity (to reflect its greater intensity) (Armstrong et al., 2000).

5.2.5 Anthropometric measurements

Anthropometric measurement methods for weight, height and WC have been previously described in the VHM report (Department of Health, 2012a).

5.2.6 Biomedical measurements

Blood collection was conducted via venepuncture after an overnight fast of ten hours or more. Blood samples were assessed for the following factors: total cholesterol, HDL-C, TG, Hba1c and FPG levels. Blood samples were centrifuged on site and were analysed at a separate central laboratory on a Siemens ADVIA 2400 Clinical Chemistry System (Siemens). Blood components were measured as following: total cholesterol using enzymatic (oxidase/peroxidase) methods; HDL-C using the elimination/catalase method; TG using GPO, Trinder with serum blank; blood glucose using the hexokinase method; and HbA1c was measured by immunoassay (Roche, Integra) (Department of Health, 2012a).

5.2.7 Blood pressure measurements

Sitting blood pressure measurements (Dinamap, 8100, GE, USA) were made in triplicate in each person, after a five minute rest period. The average of the two closest measurements (<10 mmHg SBP and <6 mmHg DBP) were used in the analysis. Further details have been reported in the VHM report (Department of Health, 2012a) .

5.2.8 Classification of MetS

MetS was classified according to the criteria from the joint interim statement of several major organizations (Alberti et al., 2009). Individuals were classified as having MetS if they had three or more of the following five components: (1) elevated TG ≥1.7 mmol/L (≥150 mg/dL); (2) reduced HDL-C concentration <1.0 mmol/L (<40 mg/dL) in males and <1.3 mmol/L (<50 mg/dL) in females; or on lipid lowering therapy (3) elevated BP (SBP ≥130 mmHg and/or DBP ≥85 mmHg) or on anti-hypertensive medications; (4) elevated FPG ≥5.6 mmol/L (≥100 mg/dL) - 6.9 mmol/L (124 mg/dL) but free of diabetes; and (5) elevated WC ≥ 94 cm for males or ≥ 90 cm for Aboriginal and Torres Strait Islander, Asian and South American males and ≥ 80 cm for females. In this analysis subjects were categorised into having or not having MetS (yes/no).

5.3 Statistical analysis

The main outcome variable was the status of MetS (yes/no). The primary exploratory variables of interest were 25OHD concentration and calcium intake which were both categorised into tertiles: low 25OHD (range 10-44 nmol/L; median 33 nmol/L), medium 25OHD (range 45-65 nmol/L; median 54 nmol/L), and high 25OHD (range 65-204 nmol/L; median 77 nmol/L), and low calcium (range 72-719 mg/d; median 579 mg), medium calcium (range 720-1009 mg/d; median 858 mg), and high calcium (range 1010-3726 mg/d; median 1233 mg). The association between all possible combinations of the 25OHD concentration and calcium intake tertiles (thereby nine levels in total) and MetS, were examined in this study, with mutual adjustment for the other components. Serum 25OHD concentration was also

tested as a continuous variable for every 10 nmol/L increment, while calcium intake was tested as a continuous variable for every 500 mg/d increment.

In the first stage of the analysis, demographic statistics and differences between the vitamin D concentration and calcium intake tertiles were tested by independent samples t-test and frequency tabulation. Furthermore, to investigate the effect of the categorical predictors of interest on the risk of having MetS and higher value of its components, a Chi-square (χ^2) test and simple binary logistic regression analysis was then conducted to obtain the crude unadjusted odds ratio and corresponding 95% CI.

Multiple logistic regression analysis was then carried out to calculate the AOR and 95% CI for the relationships between 25OHD or calcium intake and having MetS. Analyses were conducted using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp. Released 2013. Armonk, NY: IBM Corp USA). Complex samples analysis was applied to adjust for the unequal selection probability due to the multistage stratified cluster-sampling procedure used in the VHM survey. Appropriate clustering and weighting variables were used to compute appropriate standard errors and CI in the Complex samples analysis procedure. A P value of less than 0.05 was accepted as statistical significance.

5.3.1 Confounders

In our analysis we considered and tested several risk modifiers, based on our experience (Markwick, Ansari, Sullivan, & McNeil, 2015) and that of others (Brenner et al., 2011; Hypponen, Boucher, Berry, & Power, 2008; Reis, von Muhlen, & Miller, 2008; Yesil & Yilmaz, 2013). Accordingly we included the following demographic factors: weight, age, gender, country of birth, income, education level, physical activity level, smoking status, and season. Dietary factors included intakes of: alcohol, dietary fibre, energy, magnesium, retinol, 25OHD concentration (calcium intake model only), and calcium intake (25OHD concentration model only). Age, weight, alcohol, dietary fibre, energy intake, magnesium, retinol, 25OHD concentration, and calcium intake, were entered into the regression model as continuous

variables. Country of birth was identified according to those born in Australia and those born overseas. Education level was categorised according to three levels: tertiary education, TAFE/diploma/certificate, or high school and less. Smoking status was assessed on the basis of three categories: current smoker, ex-smoker, and non-smoker. Income levels were categorised according to four categories: ≥$70,000, $30,001-$70, 000, <$30,000, and don't know/refused. Season of biomedical examination were categorised as summer, autumn, winter and spring.

5.3.2 Rationale of analysis

In this analysis we examined the relationship of 25OHD concentration and calcium intake on MetS through a series of questions that resulted in different models. What was the unadjusted relationship between 25OHD and calcium intake with MetS? (**Crude model**). What was the confounding influence of socio-demographic factors on the relationship of 25OHD/calcium intake with MetS? (**Model 1**). What was the potential influence of dietary factors on the relationship of 25OHD/calcium intake with MetS? (**Model 2**).

5.4 Results

The present study population consisted of a total of 3,404 adults with a mean age of 49 years. The overall prevalence of MetS was 21.6%, with a larger proportion of males (22%) having MetS than females (14%) ($p<0.001$). The mean serum 25OHD concentration of those with MetS was 49.6 nmol/L, which was significantly lower than that for those without MetS which was 57.5 mmol/L ($p<0.001$). The mean dietary calcium intake was 849 mg in those with MetS and 926 mg in those without MetS ($p<0.001$) (Table 5.1).

Table 5.1 Demographic and clinical characteristics by the presence/absence of metabolic syndrome (MetS) among non-diabetic adults (n 3404) aged 18–75 years from the Victorian Health Monitor survey, May 2009–April 2010.

Characteristic	Absence of MetS (n=2,669)			Presence of MetS (n=735)			P value
	n (%)	mean	SE	n (%)	mean	SE	
Age (y)		41	0.9		52	1.0	<0.001
Weight (kg)		75.7	0.5		91.6	0.8	<0.001
Gender							<0.001
Males	1237 (78)		2.0	344 (22)		2.0	
Females	1565 (86)		1.1	257 (14)		1.1	
Country of birth							0.541
Born in Australia	2139 (82)		1.4	454 (18)		1.4	
Born overseas	658 (81)		2.0	153 (19)		2.0	
Education level							<0.001
Tertiary education	1136 (87)		1.5	170 (13)		1.5	
TAFE/ diploma/ certificate	580 (80)		2.3	140 (20)		2.3	
High school or less	1075 (78)		1.7	303 (22)		1.7	
Income							0.098
≥$70, 001	1341 (84)		1.4	250 (16)		1.4	
$30, 001 - $70, 000	837 (79)		2.1	220 (21)		2.1	
<$30, 000	456 (81)		2.6	110 (19)		2.6	
Don't know/refused	157 (82)		3.8	33 (18)		3.8	
Physical activity level							0.011
Sufficient physical activity (≥150 min/wk)	1929 (83)		1.5	384 (17)		1.5	
Insufficient physical activity (<149 min/wk)	704 (81)		1.9	168 (19)		1.9	
Inactive (0 min/wk)	153 (71)		4.8	62 (29)			
Smoking status							<0.001

Characteristic	Absence of MetS (n=2,669)			Presence of MetS (n=735)			P value
Current smoker	423 (80)		2.5	109 (20)		2.5	
Ex-smoker	694 (75)		2.1	224 (25)		2.1	
Non-smoker	1669 (85)		1.4	283 (15)		1.4	
Season of biomedical examination							0.182
Summer	155 (75)		5.2	52 (25)		5.2	
Autumn	677 (84)		2.0	131 (16)		2.0	
Winter	964 (84)		1.8	182 (16)		1.8	
Spring	991 (80)		2.8	252 (20)		2.8	
Vitamin D status							
Serum 25OHD (nmol/L)		57.5	2.1		49.6	2.1	<0.001
25OHD tertiles							<0.001
Low 25OHD (33 nmol/L)	857 (77)		2.1	252 (23)		2.1	
Medium 25OHD (54 nmol/L)	925 (80)		2.3	237 (21)		2.3	
High 25OHD (77 nmol/L)	1013 (89)		1.3	120 (11)		1.3	
Dietary variables							
Dietary calcium intake (mg)		926.0	11.4		849.0	20.0	<0.001
Calcium tertiles							0.001
Low calcium intake (579 mg)	847 (77)		1.8	245 (23)		1.8	
Medium calcium intake (858 mg)	908 (83)		1.4	187 (17)		1.4	
High calcium intake (1233 mg)	924 (85)		1.5	163 (15)		1.5	
Total energy intake (kJ)		9768	134		9442	163	0.021
Alcohol (g)		12.3	0.7		15.3	0.9	<0.001
Dietary fibre (g)		26.5	0.4		25.3	0.5	0.006
Magnesium (mg)		418.9	6.6		397.2	7.6	0.008
Retinol (ug)		433.8	23.2		454.5	56.1	0.428
Metabolic components							
Waist circumference (cm)		86.4	0.7		102.5	0.7	<0.001

Characteristic	Absence of MetS (n=2,669)		Presence of MetS (n=735)		P value
Fasting plasma glucose (mmol/L)	4.9	0.02	5.5	0.04	<0.001
HDL (mmol/L)	1.5	0.02	1.2	0.02	<0.001
Triglycerides (mmol/L)	1.1	0.02	2.1	0.06	<0.001
SBP (mmHg)	122	0.7	136	0.9	<0.001
DBP (mmHg)	71	0.5	81	0.4	<0.001

Data are presented as mean estimate (weighted) (%) for categorical variables, and mean estimate (weighted) and (SE) for continuous variables. Difference in the continuous and categorical variables between groups were assessed by independent samples t-test and Chi-square test, respectively.

Footnotes: MetS, metabolic syndrome; SE, standard error; min, minutes; wk, week.

5.4.1 Association between tertiles of serum 25OHD concentration, calcium intake, and presence of MetS

Every 10 nmol/L increment in 25OHD concentration reduced the likelihood of having MetS by 15% (Model 2) (Table 5.2). The Crude Model indicated that those with the highest 25OHD tertile had a 60% lower odds of having MetS. After adjusting for socio-demographic variables (Model 1), the significant inverse association between serum 25OHD concentration and presence of MetS remained. After adjustment for dietary variables (alcohol, dietary fibre, energy, magnesium, calcium and retinol), subjects with the highest 25OHD tertile had a 65% lower odds of having MetS compared to those with the lowest 25OHD tertile (Model 2) (Table 5.2).

Table 5.2 Odds ratio of having MetS by tertiles of serum 25OHD concentration.

	Crude Model		Model 1		Model 2	
	COR	95% CI	AOR	95% CI	AOR	95% CI
25OHD continuous (10 nmol/L)	0.87	0.82, 0.92	0.82	0.78, 0.85	0.85	0.80, 0.89
P value		<0.001		<0.001		<0.001

	Crude Model		Model 1		Model 2	
25OHD tertiles						
Low 25OHD (33 nmol/L) †	1.0		1.0		1.0	
Medium 25OHD (54 nmol/L) †	0.87	0.66, 1.14	0.69*	0.52, 0.90	0.77	0.58, 1.04
High 25OHD (77 nmol/L) †	0.40*	0.29, 0.56	0.29*	0.22, 0.38	0.35*	0.26, 0.48
P value for trend	<0.001		<0.001		<0.001	

Model 1: age, gender, country of birth, income, education, smoking, season.

Model 2: Model 1 plus energy intake, physical activity level, body weight, alcohol, dietary fibre, magnesium, calcium and retinol.

Footnotes: COR, crude odds ratio; AOR, adjusted odds ratio; 1.0, lowest 25OHD tertile served as the reference group; *, significant in comparison to reference group at 5% significance level; †, median of the tertile group.

Table 5.3 shows that every 500 mg increment in dietary calcium intake reduced the likelihood of having MetS by 25% after adjusting for socio-demographic variables in Model 1, but the reduction became non-significant after adding dietary variables (alcohol, dietary fibre, energy, magnesium and 25OHD concentration) (Model 2). If we did not control for 25OHD in the latter model, the AOR approached significance (AOR 0.81, 95% CI 0.64, 1.02; p=0.073) but was non-significant on controlling for 25OHD (Table 3 Model 2, p=0.141). Those with highest tertile of dietary calcium intakes had significantly reduced odds of having MetS by 39% in the Crude Model, and 37% in Model 1 in comparison to those who had the lowest tertiles of dietary calcium intake, however the comparison was not significant when dietary factors were added to Model 2 (Table 5.3). Based on previous evidence we tested for potential interactions between 25OHD concentration, calcium intake and age, gender, smoking status, physical activity, county of birth and education level, however there were no significant

interactive effects found (Daly et al., 2012). Furthermore interactions between 25OHD, calcium and dietary variables (alcohol, dietary fibre, energy, magnesium and retinol) were tested though none were significant.

Table 5.3 Odds ratio of having MetS by tertiles of dietary calcium intake.

	Crude Model		Model 1		Model 2	
	COR	95% CI	AOR	95% CI	AOR	95% CI
Calcium intake continuous (500 mg)	0.74	0.62, 0.89	0.75	0.61, 0.91	0.81	0.66, 1.06
P value		0.002		0.004		0.141
Calcium tertiles						
Low calcium (579 mg) †	1.0		1.0		1.0	
Medium calcium (858 mg) †	0.71*	0.56, 0.90	0.73*	0.56, 0.96	0.92	0.63, 1.33
High calcium (1233 mg) †	0.61*	0.46, 0.81	0.63*	0.46, 0.86	0.83	0.56, 1.21
P value for trend	0.002		0.012		0.613	

Model 1: age, gender, country of birth, income, education, smoking, season.

Model 2: Model 1 plus energy intake, physical activity level, body weight, alcohol, dietary fibre, magnesium, and 25OHD concentration.

Footnotes: COR, crude odds ratio; AOR, adjusted odds ratio; 1.0, lowest calcium tertile served as the reference group; *, significant in comparison to reference group at 5% significance level; †, median of the tertile group.

5.4.2 Association between combined effects of serum 25OHD concentration and calcium intake and presence of MetS

In view of no significant interaction between the 25OHD status and calcium intake (p=0.651) the regression analysis was extended to examine the effect of combining 25OHD concentration and calcium intake tertiles on MetS (Figure 5.1). The combination of low

25OHD tertile (median 33 nmol/L) and low calcium intake tertile (median 579 mg) was the reference group. After controlling for confounding factors, the combination of the high-25OHD and low, medium or high calcium intake significantly reduced the odds of having MetS by 72%, 70% and 66% respectively (Figure 5.1).

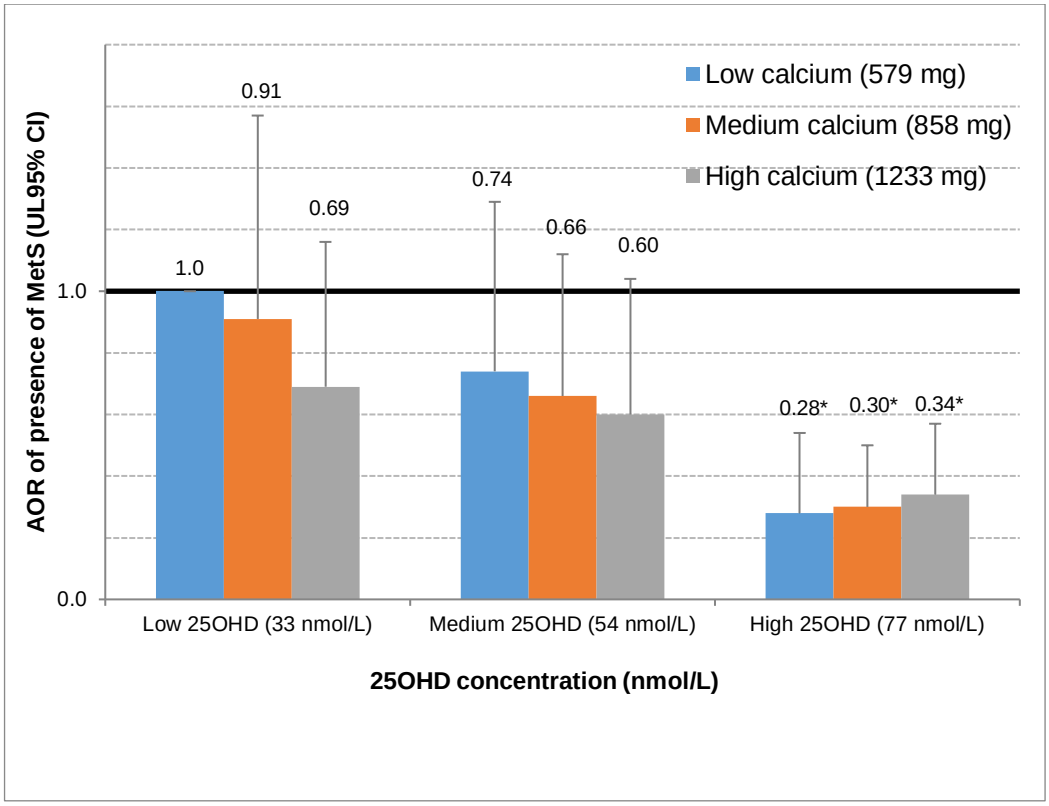

Figure 5.1 Combined effects of serum 25OHD concentration and dietary calcium intake on the presence of MetS.

Footnotes: Adjusted odds ratios (AOR), with the upper limit (UL) of the 95 % confidence interval represented by vertical bars, adjusted for age, gender, country of birth, income, education, smoking, season, physical activity level, weight, alcohol, dietary fibre, magnesium, retinol and energy intake. 'Ref.' indicates that the lowest 25OHD and lowest calcium tertile served as the reference group; *significant in comparison to reference group at 5 % significance level.

5.5 Discussion

We investigated the individual and combined association of 25OHD concentration with dietary calcium intake on MetS. In addition to many confounders, we controlled for calcium intake in the 25OHD model and for 25OHD concentration in the calcium model, to investigate their effect, independent of each other. The results of this representative sample of adults from an Australian state have indicated that higher serum 25OHD concentration per se was associated with significantly reduced odds of MetS (Table 5.2). However this was not statistically significant for every model of calcium intake tested (Table 5.3). As a continuous variable the overall pattern for calcium was in the same direction as 25OHD, and with a lower AOR (Table 5.3). If we did not control for 25OHD in the calcium intake continuous model, the AOR approached significance (AOR 0.81, 95% CI 0.64, 1.02; p=0.073) but on controlling for 25OHD (Table 5.3 Model 3, p=0.141), this was non-significant. Such outcomes suggest that prevailing 25OHD concentrations could modulate the potential effect of calcium on MetS.

Our findings are consistent with other cross-sectional and prospective studies where an inverse association between 25OHD concentration, calcium intake and MetS were observed (Brenner et al., 2011; Gagnon et al., 2012; Gradillas-García, Álvarez, Rubio, & de Abajo, 2015; Hypponen et al., 2008; Liu et al., 2005; Vitezova et al., 2015). One cross-sectional study found a 67% reduction in the odds of having MetS in those with the highest 25OHD tertile (68-231 nmol/L), versus the lowest tertile (9-45 nmol/L) (Hypponen et al., 2008). Our study obtained relatively similar results where the highest tertile of 25OHD was found to contribute a 65% reduced odds for MetS in comparison to the lowest tertile (Table 5.2). The study by Hypponen et al. (2008), had twice the sample size but only adjusted for gender, month and hour of blood measurement. In comparison we controlled for additional socio-demographic, anthropometric and dietary covariates. A more recent prospective study in the elderly also found an inverse association between MetS and high 25OHD (≥75 nmol/L), though the magnitude of their findings was much lower (Vitezova et al., 2015). Furthermore, a large prospective study reflected our results and found a 36% reduction in odds of having MetS in

the highest calcium intake group (1005-2596 mg/d) in comparison to the lowest calcium group. Overall despite differences between such studies in sample sizes, study design (cross-sectional vs. prospective), age of subjects and confounders used, the protective effect of vitamin D in reducing the odds of having MetS, appears consistent.

We also examined the potential additive effects of tertile combinations of 25OHD concentration and calcium intake on MetS (Figure 5.1). The outcomes were interesting since they suggested that at low and medium tertiles of 25OHD, there was a trend for increasing calcium intake to reduce AOR of MetS (Figure 5.1). However, in the highest 25OHD tertile this trend disappeared with significantly reduced AORs across the range from low to high calcium intakes. This was suggestive of a plateau effect, raising the possibility of a threshold to the interplay between calcium and 25OHD on functional outcomes.

It is now well known that increasing calcium intake increases passive calcium absorption from the GI tract (Heaney, 2008). A higher calcium intake also increases the half-life of 25OHD in circulation (Lips, 2012) and together these actions may explain the effect of high calcium in the lowest 25OHD tertile (Figure 5.1). However, a key physiological function of 25OHD is the maintenance of calcium homeostasis via active intestinal calcium absorption (Heaney, 2008; Norman, 1990; Shapses et al., 2012). So an improvement in vitamin D status from the low to medium tertile (Figure 5.1), would further increase active calcium absorption and possibly allow for a greater effect of calcium on MetS. In support of such a paradigm, was the observation that the overall effect of calcium in the medium 25OHD tertile was stronger in comparison to the low 25OHD tertile (Figure 5.1). While 25OHD and calcium absorption have a positive relationship, there is a plateau to this effect. Above ~80 nmol/L, active calcium absorption does not respond to further increases in 25OHD (Heaney, 2008). It is notable that the latter concentration falls within the highest tertile of 25OHD in this study, and may explain why increasing calcium intake ceases to have any added benefit in highest tertile (Figure 5.1).

There is another related and important facet to these relationships. A raised PTH concentration is associated with an increased the risk of MetS (Ahlström et al., 2009; Huang et al., 2013; Soares et al., 2011b). Increases in dietary calcium and in 25OHD would lower circulating PTH. Recent data has described the exponential decline in PTH with increases in 25OHD (Durazo-Arvizu et al., 2010). The analysis indicated two inflection points in the relationship, with the second plateau at 25OHD concentrations above ~70 nmol/L where PTH was maximally suppressed (Durazo-Arvizu et al., 2010). We acknowledge that this threshold value of 25OHD is not universally accepted (Lucas & Neale, 2014) and that further work is necessary. However it serves the argument that at the highest tertile of 25OHD in this study, the negative effects of a raised PTH on MetS could be significantly diminished relative to the previous tertiles. Overall, our results argue that calcium intake has an added effect with 25OHD on reducing MetS, but this only applies up to the medium tertile of 25OHD (Figure 5.1). Above the latter the observed effects are mainly due to 25OHD per se. There is some evidence in the literature in support of threshold effects, especially for outcomes that impinge on MetS. A RCT has demonstrated that following vitamin D supplementation, significant increases in insulin sensitivity (HOMA%S) were only observed in those who achieved a 25OHD concentration of 80 nmol/L and had maintained that value for six months (von Hurst et al., 2010). In a weight loss RCT, participants who achieved 80 nmol/L at 12 months, demonstrated significantly greater losses in weight, percent fat mass and WC, compared to those who did not (Mason et al., 2014). We cannot predict the threshold value of 25OHD from our study. Moreover, as the outcomes of these RCTs were derived from post-hoc analyses, they only support the hypothesis, rather than validate an 80 nmol/L cut-off.

5.5.1 Potential mechanisms

There are many mechanistic pathways to support our observations of a protective effect of 25OHD concentrations on MetS. An animal study suggests an independent effect of 25OHD on beta cells, with improvements in impaired glucose tolerance and insulin secretion, despite prevailing plasma calcium concentrations (Cade & Norman, 1986). $1,25(OH)_2D$ has a role in

insulin secretion (Cavalier et al., 2011), where it stimulates the expression of the insulin receptor and increases the responsiveness to glucose transport. During vitamin D deficiency, beta cell function is inhibited leading to a decrease in insulin secretion (Norman, Frankel, Heldt, & Grodsky, 1980). In addition, inadequate 25OHD concentration is associated with IR (Mathieu, Gysemans, Giulietti, & Bouillon, 2005; Palomer, Gonzalez-Clemente, Blanco-Vaca, & Mauricio, 2008; Procopio & Borretta, 2003). While we acknowledge that IR does not always explain all of MetS (Cozzolino, Ketteler, & Zehnder, 2010; Merke et al., 1989; Vaidya, Forman, & Williams, 2011), it is a key feature in the pathophysiology of the syndrome (Nasser, 2009). The nVDR and 1-α hydroxylase enzyme is found in tissues not related to calcium metabolism, such as in cardiac myocytes, endothelial and smooth vascular muscle cells (Merke et al., 1989); potentially underscoring a role of 25OHD in cardiovascular health. The RAS is important in the regulation of BP (Schmieder, Hilgers, Schlaich, & Schmidt, 2007) and low 25OHD concentration may dysregulate the control of RAS (Vaidya et al., 2011). In this context lower 25OHD concentration has been found to be inversely correlated with measures of arterial stiffness, and also to increased arterial resistance, hypertension and endothelial dysfunction (Alyami, Soares, Sherriff, & Mamo, 2014; Giallauria et al., 2012; Lee et al., 2012; Ullah, Uwaifo, Nicholas, & Koch, 2010). Moreover higher vitamin D status could also reduce islet beta cell damage by reducing islet RAS activity, thereby reducing the risk of hyperglycaemia (Leung, 2016).

The beneficial effect of calcium on features of MetS, may arise from both its absorbed fraction and its unabsorbed fraction in the GI tract (Soares et al., 2012). There is now increasing evidence that calcium intake may influence fat balance and hence energy balance. Dietary calcium increases whole body fat oxidation and this could, potentially, reduce circulating fatty acids/lipids (Gonzalez et al., 2012; Soares et al., 2012). Unabsorbed calcium is not without metabolic effects (Soares et al., 2012). A meta-analysis indicates that for ~1200 mg/d of dairy calcium intake an increase of ~5 g/d in faecal fat can be expected (Christensen et al., 2009). This arises from the interaction of non-absorbed calcium and dietary fat in the GI

lumen leading to calcium-fatty acid soap formation and hence its eventual excretion. These outcomes may contribute to lower circulating TG and other lipid fractions seen with calcium supplementation (Major et al., 2007). Finally, as with other chronic non-communicable conditions, MetS is a low-grade chronic inflammatory state. We, and others, are of the opinion that adequate vitamin D has a significant role in ameliorating the inflammatory state in chronic disease (Calton, Keane, Newsholme, & Soares, 2015a; Calton et al., 2015b; Lai & Fang, 2013).

5.6 Limitations

The cross-sectional design has only permitted an examination of associations between calcium intake, vitamin D status and MetS. Though we have controlled for recognised confounders, we cannot establish which came first, lower 25OHD concentration and calcium intake or having MetS. An increased requirement for these nutrients in chronic conditions like MetS is a possibility and may account for a reverse causation. Unlike some European countries, there is no mandatory fortification of the Australian food supply for these nutrients. Unfortunately this survey did not include information on calcium and vitamin D supplement usage. That information would have potentially allowed us to tease out the effect of food derived calcium and sunlight derived vitamin D status (since vitamin D in Australian foods is low) vs. pharmacological intake. We however, approached the potential confounding effect of supplement calcium intake using random generated surrogate data for different age groups, based on the calcium supplement intake percentages collected in the Australian Health Survey 2011-12 (Australian Bureau of Statistics, 2011-12). We found that the changes between crude and adjusted effect estimates was much less than 10%; a cut-off criterion for being a sizable confounder in epidemiology research (Kurth & Sonis, 2007). Hence, we do not anticipate significant confounding by supplement derived calcium intake on the association between dietary calcium intake and the risk of MetS in this study.

Serum 25OHD can be affected by genetic variation of the major transporter, the DBP (Boucher, 2012; Lauridsen et al., 2005; Speeckaert, Huang, Delanghe, & Taes, 2006). This is

seen as variations in DBP concentration (Boucher, 2012; Fu et al., 2009; Lauridsen et al., 2005) as well as DBP phenotypes potentially having stronger binding abilities than others (Arnaud & Constans, 1993). Serum 25OHD can also differ due to genetic variation in its key activation enzyme CYP27B1 (Hypponen, Berry, Wjst, & Power, 2009), that converts 25OHD to the active form. Such genetic variant information was not collected in the VHM survey so is a potential confounding factor. Future studies in this area could include this information to provide a more complete picture.

A small proportion of our sample were from South Asia (1.6%, n=56), an ethnic group associated with high rates of betel nut chewing. Chewing betel nut could increase the risk of developing T2DM (Tseng, 2010) and animal studies have indicated that betel nut ingestion in male parents may contribute to inheritable glucose intolerance in their offspring (Boucher, Ewen, & Stowers, 1994). Such data are not available for Australia and was not collected as part of the VHM survey. However, exclusion of these cases (n=56) did not change the direction or magnitude of our results. We therefore anticipate minimal confounding from such a potential habit in our South Asian participants.

5.7 Strengths

We have used a large representative population based sample of one Australian state that covered an age range 18-75 years. The dietary data was collected through a multiple-pass 24 hour dietary recall which is the current standard and all blood analysis was conducted centrally by one laboratory based on standard methodology. Our analysis has considered and adjusted for many socio-demographic and nutrient confounders, with further adjustment for energy intake. We acknowledge that this field of research would benefit from the confirmation of a causal role for calcium and vitamin D in MetS. While RCTs provide Level 1 evidence, they are not necessarily the mainstay of the evidence base for public health nutrition, and in deciding nutrition priorities for better health (Mann, 2002; Truswell, 2001).

5.8 Conclusion

The study demonstrates that a high 25OHD concentration was associated with significant reductions in the odds of MetS. We raise the possibility that the benefit of calcium is restricted to low and medium 25OHD concentrations, and this may represent a threshold to the interplay between calcium and 25OHD on functional outcomes. Overall, these population based results contribute to the evidence in favour of a role for vitamin D and calcium in modulation of metabolic syndrome risk.

Chapter 6 Vitamin D status, dietary calcium intake and the components of the metabolic syndrome

The content of this chapter is covered by a recently accepted paper.
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Pannu, P. K., Soares, M. J., Piers, L. S., Zhao, Y., & Ansari, Z. (2017). The association of vitamin D status and dietary calcium intake with individual componenets of the metabolic syndrome: a population based study in Victoria, Australia. Submitted to: *Cardiovascular Endocrinology*, 23/05/2017. Manuscript ID: CAEN-D-17-00009.

Objective addressed

Objective 4b: To determine whether vitamin D status, dietary calcium intake and their combination were associated with individual components of MetS.

6.1 Introduction

The MetS is the clustering of five inter-related biomedical components including central adiposity, hypertension, hypertriglyceridemia, low HDL-C and altered glucose homeostasis (Alberti et al., 2009). It is an increasingly important health issue with approximately 25% of the global population being diagnosed with having MetS (Cameron et al., 2004). The diagnosis of MetS predicts an increased risk of T2DM, CVD morbidity and mortality (Eckel, Alberti, Grundy, & Zimmet, 2010). Furthermore, the risk of T2DM appears to increase in line with the presence of increasing MetS components with an increased risk of 11.9%, 31.2% and 40.8% in those with zero, three and four or more MetS components respectively (Wannamethee, Shaper, Lennon, & Morris, 2005). However, each MetS component is an independent risk factor that clinicians will treat if diagnosed in their patients.

Vitamin D deficiency is a worldwide health issue, and an estimated 1 billion individuals are classified as having insufficient vitamin D status (Holick & Chen, 2008; Lips, 2010). Coupled with this are lower intakes of calcium (Ca) rich foods, despite increasing production and availability of dairy products (Wang & Li, 2008). 25OHD has been implicated in the mechanisms underlying a number of the MetS components (Holick, 2007). The majority of studies have found an inverse relationship between 25OHD and glucose homeostasis, lipids, obesity and blood pressure (BP) (Holick, 2007; Martini & Wood, 2006). However, a few studies have also shown an inverse relationship with Ca intake and MetS (Cho et al., 2009; Liu et al., 2005), with limited evidence supporting an association between Ca intake and individual MetS components. Vitamin D and Ca are physiologically tightly inter-linked and it is possible that their combination may have a greater potential to interactively influence effects on components of Mets (Liu et al., 2005; Pannu, Zhao, Soares, Piers, & Ansari, 2016c). We previously found that in individuals within low to medium 25OHD tertiles, increasing Ca intake tertiles had a trend to lower AOR of MetS. However in the high 25OHD tertile, this trend was no longer present (Pannu et al., 2016c). The aim of this study was to explore the

potential combined effect of vitamin D status and dietary Ca intake on individual components of MetS.

6.2 Methods

This cross-sectional study was based on the analysis of the VHM survey, which is a population based survey of Victorian adults aged 18-75 years. Details about the VHM, and the collection and analysis of the physical, dietary and biomedical variables can be found in Chapter 3 and previous publications (Department of Health, 2012a, 2012b; Pannu et al., 2016c).

6.2.1 Sample

In this sample, participants were excluded if: there was missing information on HbA1c data (n=31), those with HbA1c ≥6.5% as they were classified as having T2DM according to the ADA cut-offs (n=39) (American Diabetes Association, 2010), those with diagnosed T2DM (n=140), those with type 1 diabetes mellitus (n=9), those on diabetic medications (n=25), and those with missing data on metabolic components (n=22). Thus, out of 3,653 participants recruited in VHM survey, this study sample consisted of a total of 3,387 participants. The three independent variables of interest, were: serum 25OHD concentration, dietary Ca intake, and the combination of 25OHD and dietary Ca.

6.2.2 Biomedical analyses

Venous blood was collected by venepuncture, processed, refrigerated and transported daily to a single accredited laboratory in Melbourne for storage (at -80°C). All analyses and reporting was completed within two weeks of collection. Blood samples were analyzed for: fasting plasma glucose (FPG) using the hexokinase method; HDL-C using elimination/catalase method; and triglycerides (TG) using GPO Trinder reagent set with serum blank. Blood pressure measurements were collected (GE Dinamap 8100 Vital Sign Monitor) after a five minute rest (Department of Health, 2012a).

Serum 25OHD was measured using the DiaSorin Corporation Liaison® 25OHD total assay, an automated direct competitive chemiluminescent immunoassay that measures D2 and D3 to provide a total value for circulating vitamin D in nmol/L. The detection limit was 10 nmol/L. The precision of the LIAISON assay was determined by using human serum-based quality controls, spanning a 25OHD range of 35–180 nmol/L. Each control sample was assayed in 2–4 replicates per run for 3–4 runs; however within-run and total CVs were not reported. The laboratory was not part of the vitamin D standardization programs at the time of fieldwork for the study (2009-10). The ALTM (All Laboratory Trimmed Mean) was not computed by the laboratory, nor were results compared with a Target Value (TV) assigned by the NIST (National Institute of Standards and Technology) Reference Measurement Procedure.

6.2.3 Dietary calcium intake

Dietary data was collected via a multiple pass 24 h diet recall using computer assisted telephone interviews. The FoodWorks® nutrition software (FoodWorks® Interview) were used in conducting the three dietary recalls (Department of Health, 2012b). This provided data on energy, macronutrient, micronutrient and Ca intake. Dietary Ca intake was included as a continuous variable for every 500 mg/d increment.

6.2.4 25OHD concentration and dietary calcium intake

25OHD concentration was categorised into three groups based on its tertiles namely; low 25OHD tertile (range 10-44 nmol/L; median 33 nmol/L), medium 25OHD tertile (range 45-65 nmol/L; median 54 nmol/L), and high 25OHD tertile (range 65-204 nmol/L; median 77 nmol/L). Dietary Ca intake were also classified based on its tertiles which were: low Ca tertile (range 72-719 mg/d; median 579 mg/d), medium Ca tertile (range 720-1009 mg/d; median 858 mg/d), and high Ca tertile (range 1010-3726 mg/d; median 1233 mg/d).

To assess the combined effect of 25OHD and Ca tertiles, a nine level variable was generated based on the combination of the established tertiles which were: 1) low 25OHD and low Ca; 2) low 25OHD and medium Ca; 3) low 25OHD and high Ca; 4) medium 25OHD and low Ca; 5) medium 25OHD and medium Ca; 6) medium 25OHD and high Ca; 7) high 25OHD and low Ca; 8) high 25OHD and medium Ca; 9) and high 25OHD and high Ca.

6.2.5 MetS components

Information on the collection of the physical and anthropometric measurements can be found in Chapter 3 and our previous publications (Department of Health, 2012a; Pannu et al., 2016c). MetS components were the dependent variables, and were categorized as per the cut-offs defined by the joint interim statement of key organizations (Alberti et al., 2009). The criteria for the MetS components were: (1) elevated TG ≥1.7 mmol/L (≥150 mg/dL) (yes/no); (2) reduced HDL-C concentration <1.0 mmol/L (<40 mg/dL) in males and <1.3 mmol/L (<50 mg/dL) in females (yes/no); or on lipid lowering therapy; (3) elevated SBP (≥130 mmHg or on anti-hypertensive medications (yes/no); (4) elevated DBP (≥85 mmHg) or on anti-hypertensive medications (yes/no); (5) elevated FPG ≥5.6 mmol/L (≥100 mg/dL) (yes/no); and (6) elevated WC ≥94 cm for males, (≥90 cm for Aboriginal and Torres Strait Islander, Asian and South American males) and ≥80 cm for females (yes/no).

6.2.6 Confounders

We adjusted for the following confounders in our analysis based on our previous findings (Pannu et al., 2016c) and as reported from others (Vitezova et al., 2015). The demographic factors included were: age (years), gender, country of birth (born in Australia, born overseas), BMI (kg/m^2), physical activity level (insufficient physical activity <150 min/week, sufficient physical activity (≥150 min/week), smoking status (current smoker, non-smoker), income (<$30,000, $30,001-$70,000, ≥$70,001, don't know/refused), and education (high school or less, TAFE/diploma/certificate, tertiary education). Season which was categorised as summer, autumn, winter, spring. The dietary variables were all entered as continuous variables and

were: energy intake (KJ/d), dietary fibre (g/d), alcohol (g/d), magnesium (mg/d), zinc (mg/d), Ca (25OHD model only), and 25OHD (Ca model only).

6.2.7 Statistical analysis

The analysis targeted the following questions:

Was there any association between MetS components and increments of 10 nmol/L of 25OHD?

Was there any association between MetS components and increments of 500 mg/d of dietary Ca intake?

Did the combination of 25OHD and Ca tertiles influence any of the MetS components?

The analysis was conducted according to the following steps:

Step 1: Demographic/descriptive statistics by the MetS components were obtained and differences between groups were tested by χ^2 test for categorical variables of interest.

Step 2: Logistic regression analysis was used and the following regression models were developed to examine if there were any association between MetS components and 1) increments in 25OHD concentration, 2) increments in dietary Ca intake, and 3) the combination of 25OHD and Ca tertiles, separately, and unadjusted OR, AOR and 95% CI were obtained and reported.

Model 1: An unadjusted logistic regression model was fitted for MetS components including 1) 25OHD concentration only, 2) Ca intake only, and 3) the combination of 25OHD concentration and Ca tertiles. The combination of 'low 25OHD and low Ca' combination tertile served as the reference group.

Model 2: We adjusted the *Model 1* for the following confounders: age, sex, country of birth, smoking status, physical activity, income, education, BMI, season, energy intake, dietary fibre, alcohol, magnesium, zinc, Ca (25OHD model only), and 25OHD (Ca model only).

Model 3: We explored if any of the associations between the combination of 25OHD and Ca tertiles and MetS components were independent from the rest of the MetS components. Thus, we adjusted *Model 2* for the respective MetS components (reduced HDL-C, elevated WC, elevated TG, elevated SBP, elevated DBP, and elevated FPG).

Analyses were conducted using the IBM SPSS Statistics for Windows, Version 21.0 'Complex Samples' module to eliminate sampling bias arising from the multistage cluster sampling method used in the VHM survey.

6.3 Results

The mean age was 49 years with a higher proportion of subjects with normal HDL-C (86%), TG (79%), SBP (58%), DBP (73%) and FPG (84%) vs. abnormal levels. Selected demographic characteristics for each MetS component are in Appendix P (Table Q.1-Q.6). Demographic and clinical characteristics by the absence/presence of MetS are presented in Table 6.1

Table 6.1 Demographic and clinical characteristics by the absence/presence of MetS.

Characteristic	Absence of MetS (n=2655)		Presence of MetS (n=732)		P value
	n (%)	Mean (SE)	n (%)	Mean (SE)	
Age (y)		41 (0.9)		52 (1.0)	<0.001
Body mass index (kg/m^2)		26.1 (0.2)		31.3 (0.2)	<0.001
Gender					<0.001
Males	1231 (78%)		345 (22%)		
Females	1556 (86%)		255 (14%)		

Country of birth					0.569
Born in Australia	2126 (82%)		454 (18%)		
Born overseas	654 (81%)		152 (19%)		
Education level					<0.001
Tertiary education	1134 (87%)		169 (13%)		
TAFE/ diploma/ certificate	575 (81%)		139 (19%)		
High school or less	1066 (78%)		302 (22%)		
Income					0.108
≥$70, 001	1332 (84%)		252 (16%)		
$30, 001 - $70, 000	832 (79%)		220 (21%)		
<$30, 000	452 (81%)		109 (19%)		
Don't know/refused	157 (83%)		33 (17%)		
Physical activity level					0.027
Sufficient physical activity (≥150 min/wk)	1920 (83%)		382 (17%)		
Insufficient physical activity (<149 min/wk)	855 (79%)		227 (21%)		
Smoking status					0.159
Current smoker	419 (80%)		108 (20%)		
Non-smoker	2361 (83%)		497 (17%)		
Vitamin D status					
Serum 25OHD (nmol/L)		57.6 (2.1)		49.6 (2.1)	0.002
25OHD tertiles					<0.001
Low 25OHD (33 nmol/L)†	851 (77%)		253 (23%)		
Medium 25OHD (54 nmol/L)†	920 (80%)		236 (20%)		
High 25OHD (77 nmol/L)†	1008 (89%)		119 (11%)		
Dietary calcium intake					
Dietary calcium intake (mg/d)		926.1 (11.3)		849.4 (19.8)	<0.001

Calcium tertiles				0.001
Low calcium intake (579 mg)/d†	844 (78%)	243 (12%)		
Medium calcium intake (858 mg/d)†	903 (83%)	187 (17%)		
High calcium intake (1233 mg/d)†	921 (85%)	158 (15%)		
Metabolic components				
WC (cm)		86.4 (0.7)	102.5 (0.7)	<0.001
FPG (mmol/L)		4.9 (0.01)	5.5 (0.03)	<0.001
HDL-C (mmol/L)		1.5 (0.01)	1.2 (0.02)	<0.001
TG (mmol/L)		1.1 (0.02)	2.1 (0.1)	<0.001
SBP (mmHg)		121.7 (0.7)	136.9 (0.9)	<0.001
DBP (mmHg)		71.4 (0.5)	81.5 (0.5)	<0.001

Footnotes: WC, waist circumference; HDL-C, high density lipoprotein cholesterol. TG, triglycerides; SBP, systolic blood pressure; DBP diastolic blood pressure; FPG, fasting plasma glucose; †, median of the tertile.

6.3.1 Association between 25OHD concentration and MetS components

The unadjusted model found a significant association between every 10 nmol/L increment in 25OHD concentration and a reduced odds of elevated WC (p=0.014), TG (p<0.001), DBP (p=0.006), FPG (p=0.027) and reduced HDL-C (p=0.017). There was no significant association between 25OHD concentration and elevated SBP (p=0.189). In Model 2, after adjustment for all confounders (age, sex, country of birth, smoking, physical activity, income, education, BMI, season, Ca, energy, fibre, alcohol, magnesium, zinc), and Model 3, after further adjustment for respective MetS components, every 10 nmol/L increment in 25OHD reduced the odds of elevated TG by 21%, and elevated FPG by 9% (Table 6.2). There was no

significant association between 25OHD concentration and elevated WC (p=0.707), elevated SBP (p=0.525) and reduced HDL-C (p=0.433) (Table 6.2).

Table 6.2 The odds ratio of having MetS components for every 10 nmol/L increment in 25OHD concentration.

	25OHD (10 nmol/L increment) Model 1			25OHD (10 nmol/L increment) Model 2			25OHD (10 nmol/L increment) Model 3		
	AOR	95% CI	P value	AOR	95% CI	P value	AOR	95% CI	P value
Elevated WC	0.93*	0.88, 0.99	0.014	0.97	0.90, 1.06	0.521	0.98	0.91, 1.07	0.707
Reduced HDL-C	0.93*	0.88, 0.99	0.017	0.93*	0.87, 0.99	0.023	0.97	0.90, 1.05	0.433
Elevated TG	0.82*	0.77, 0.87	<0.001	0.79*	0.74, 0.84	<0.001	0.79*	0.74, 0.84	<0.001
Elevated SBP	0.97	0.93, 1.01	0.189	1.01	0.96, 1.05	0.961	1.02	0.97, 1.07	0.525
Elevated DBP	0.94*	0.89, 0.98	0.006	0.96	0.92, 1.00	0.050	0.97	0.92, 1.01	0.147
Elevated FPG	0.92*	0.86, 0.99	0.027	0.91*	0.86, 0.97	0.003	0.91*	0.86, 0.96	0.002

Model 1 unadjusted model.

Model 2 adjusted for: age, sex, country of birth, smoking, physical activity level, income, education, BMI, season, calcium, energy, fibre, alcohol, magnesium, zinc.

Model 3 adjusted for all variables in Model 2 and remaining MetS components.

Footnotes: BMI, body mass index; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglycerides; WC, waist circumference; *significant as compared to the reference category.

6.3.2 Association between dietary calcium intake and MetS components

The unadjusted model found a significant association between every 500 mg/d increment in dietary Ca intake and reduced odds of elevated WC (p=0.008), elevated DBP (p<0.001), and reduced HDL-C (p<0.001). There were no significant associations between dietary Ca intake and elevated TG (p=0.540), elevated SBP (p=0.208), and elevated FPG (p=0.142). After

adjustment for confounders in Model 2 and 3, every 500 mg/d increment in dietary Ca intake reduced the odds of elevated DBP by 20% (Table 6.3). There were no significant associations between increments of 500 mg/d in dietary Ca intake and elevated WC (p=0.709), elevated TG (p=0.635), and elevated FPG (p=0.907) (Table 6.3).

Table 6.3 The odds ratio of having MetS components for every 500 mg/d increment in dietary Ca intake.

	Dietary calcium (500 mg/d increment) Model 1			Dietary calcium (500 mg/d increment) Model 2			Dietary calcium (500 mg/d increment) Model 3		
	AOR	95% CI	P value	AOR	95% CI	P value	AOR	95% CI	P value
Elevated WC	0.83*	0.72, 0.95	0.008	0.94	0.70, 1.26	0.672	0.95	0.70, 1.28	0.709
Reduced HDL-C	0.64*	0.50, 0.80	<0.001	0.82	0.61, 1.10	0.174	0.80	0.61, 1.05	0.104
Elevated TG	0.95	0.79, 1.14	0.540	1.02	0.82, 1.26	0.870	1.05	0.84, 1.31	0.635
Elevated SBP	0.91	0.80, 1.05	0.208	0.86	0.71, 1.04	0.113	0.86	0.72, 1.03	0.099
Elevated DBP	0.76*	0.65, 0.88	<0.001	0.81*	0.66, 0.99	0.044	0.80*	0.66, 0.99	0.038
Elevated FPG	0.87	0.72, 1.05	0.142	0.97	0.76, 1.25	0.824	0.99	0.77, 1.26	0.907

Model 1 unadjusted model.

Model 2 adjusted for: age, sex, country of birth, smoking, physical activity level, income, education, BMI, season, 25OHD, energy, fibre, alcohol, magnesium, zinc.

Model 3 adjusted for all variables in Model 2 and remaining MetS components.

Footnote: BMI, body mass index; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglycerides; WC, waist circumference; *significant as compared to the reference category.

6.3.3 Association between the combination levels of 25OHD concentration and dietary Ca intake and MetS components

Model 1 (Appendix P, Table P.7) and Model 2 (Appendix P, Table P.8) revealed a significant association between the combination of 25OHD concentration and Ca intake and

reduced HDL-C, elevated TG and elevated DBP. After further adjustment for respective MetS components in Model 3, the significant association remained between the combination of 25OHD concentration and Ca intake and reduced HDL-C, and elevated TG. There was no significant association between combined 25OHD and Ca intake and elevated DBP, WC, SBP and FPG (Table 6.4).

6.3.3.1 The combined effects of 25OHD concentration and dietary Ca intake on reduced HDL-C

In all models (Appendix P, Table P.7; Appendix P, Table P.8; Figure 6.1, Table 6.3), the significant association persisted between the combination of 25OHD concentration and Ca intake and reduced HDL-C. In all models, and all nine combinations of 25OHD concentration and Ca intake, there appeared to be a downward trend, with a lower AOR of reduced HDL-C, as Ca intake increased. There appeared to be a dose-response effect of Ca at low 25OHD on reduced HDL-C, but at higher 25OHD, these effects of calcium were blunted. In Model 3, the following four combinations appeared to lower the odds of reduced HDL-C by: 47% in low 25OHD and medium Ca; 68% in medium 25OHD and high Ca, 57% in medium 25OHD and low Ca, and 68% in medium 25OHD and high Ca (Figure 6.1, Table 6.4).

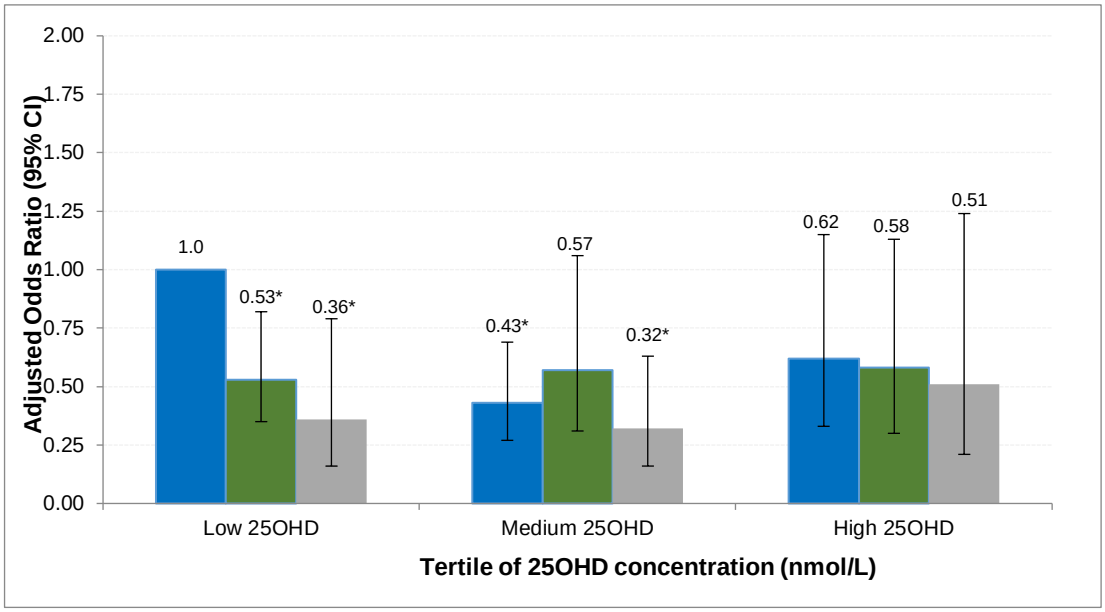

Figure 6.1 Model 3: The AOR of 25OHD tertiles and dietary calcium intake tertiles on reduced HDL-C.

Footnote: *significant as compared to the reference category.

Figure 6.1 adjusted for: age, sex, country of birth, smoking, physical activity, income, education, BMI, season, energy, fibre, alcohol, magnesium, zinc, and MetS components.

6.3.3.2 The combined effects of 25OHD concentration and dietary Ca intake on elevated TG

In all models the significant association persisted between the combination of 25OHD concentration and Ca intake and elevated TG. The combination of medium 25OHD and low and, medium Ca, significantly reduced the odds of having elevated TG by 43% and 41% respectively ($p<0.001$). The combination of high 25OHD and low, medium, and high Ca significantly reduced the odds of having elevated TG by 83%, 62%, and 67% respectively ($p<0.001$) (Figure 6.2, Table 6.4).

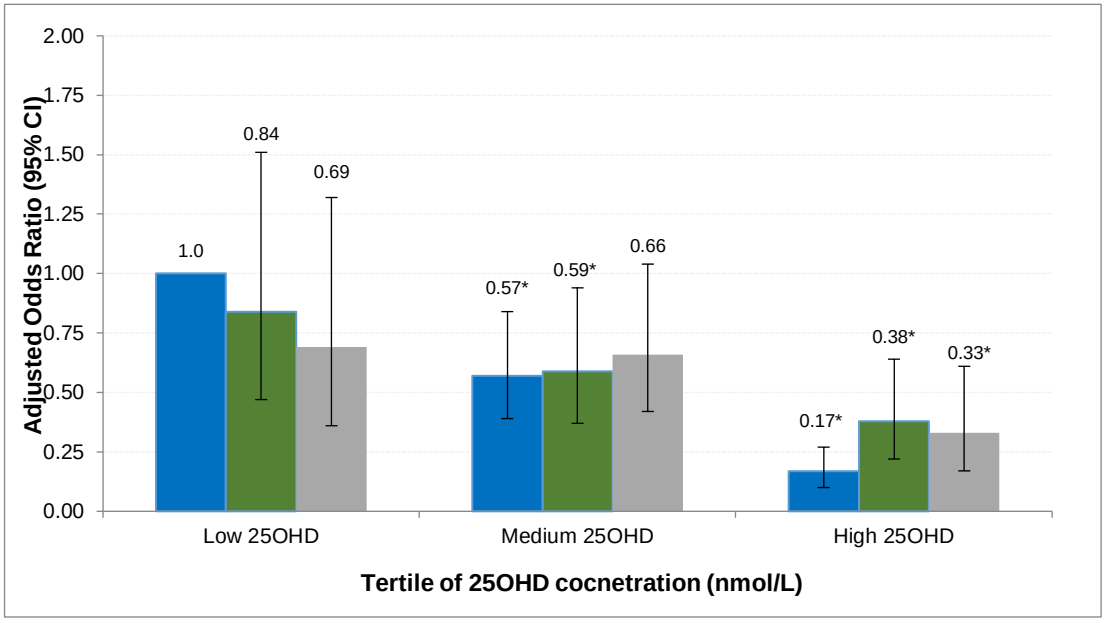

Figure 6.2 Model 3: The AOR of 25OHD tertiles and dietary calcium intake tertiles on elevated TG.

Footnote: *significant as compared to the reference category.

Figure 6.2 adjusted for: age, sex, country of birth, smoking, physical activity, income, education, BMI, season, energy, fibre, alcohol, magnesium, zinc, and MetS components.

Table 6.4 Model 3: Adjusted odds ratio of having MetS components by combination of tertiles of 25OHD and tertiles of dietary calcium intake.

	Low 25OHD (33 nmol/L)†		Medium 25OHD (54 nmol/L)†		High 25OHD (77 nmol/L)†		
	AOR	95% CI	AOR	95% CI	AOR	95% CI	P value
Low Ca (579 mg)†							
Elevated WC	1.0		1.16	0.47, 2.87	1.21	0.52, 2.79	0.999
Reduced HDL-C	1.0		0.43*	0.27, 0.69	0.62	0.33, 1.15	0.035
Elevated TG	1.0		0.57*	0.39, 0.84	0.17*	0.10, 0.27	<0.001
Elevated SBP	1.0		1.22	0.84, 1.79	0.93	0.56, 1.54	0.133
Elevated DBP	1.0		0.95	0.50, 1.80	1.06	0.63, 1.78	0.080
Elevated FPG	1.0		1.20	0.57, 2.54	0.55	0.28, 1.06	0.105
Medium Ca (858 mg)†							
Elevated WC	1.09	0.59, 2.01	1.05	0.60, 1.85	0.95	0.43, 2.09	0.999
Reduced HDL-C	0.53*	0.35, 0.82	0.57	0.31, 1.06	0.58	0.30, 1.13	0.035
Elevated TG	0.84	0.47, 1.51	0.59*	0.37, 0.94	0.38*	0.22, 0.64	<0.001
Elevated SBP	0.96	0.54, 1.70	0.90	0.52, 1.57	1.14	0.66, 1.98	0.133
Elevated DBP	0.99	0.56, 1.76	0.83	0.47, 1.48	0.57	0.39, 0.85	0.080
Elevated FPG	1.08	0.65, 1.79	0.72	0.40, 1.30	0.78	0.40, 1.52	0.105
High Ca (1233 mg)†							
Elevated WC	1.06	0.50, 2.24	1.09	0.49, 2.45	0.97	0.50, 1.89	0.999
Reduced HDL-C	0.36*	0.16, 0.79	0.32*	0.16, 0.63	0.51	0.21, 1.24	0.035
Elevated TG	0.69	0.36, 1.32	0.66	0.42, 1.04	0.33*	0.17, 0.61	<0.001
Elevated SBP	0.75	0.41, 1.39	0.80	0.48, 1.33	0.69	0.37, 1.28	0.133
Elevated DBP	0.90	0.48, 1.71	0.81	0.49, 1.35	0.67	0.34, 1.33	0.080
Elevated FPG	1.18	0.56, 2.51	0.71	0.37, 1.39	0.58	0.28, 1.20	0.105

Model adjusted for: age, sex, country of birth, smoking, physical activity level, income, education, BMI, season, energy, fibre, alcohol, magnesium, zinc, MetS components.

Footnote: Body mass index, BMI; Ca, calcium; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglycerides; WC, waist circumference; †Median 25OHD concentration and dietary calcium intake in each tertile; *significant as compared to the reference category.

6.4 Discussion

In this study we investigated the associations between the individual MetS components and 1) 25OHD concentration, 2) dietary Ca intake, and 3) the combination of 25OHD concentration and dietary Ca intake, since they are commonly recommended together for optimal bone health. After adjustment for demographic, physical, dietary factors and MetS components, every 10 nmol/L increment in 25OHD reduced the odds of elevated TG and elevated FPG. Every 500 mg/d increment in dietary Ca intake reduced the odds of elevated DBP. The combination of 25OHD concentration and Ca intake lowered the odds of having low HDL-C, and elevated TG.

6.4.1 Previous studies of 25OHD and MetS components

We found that increments of 10 nmol/L in 25OHD lowered the odds of having elevated TG, and having elevated FPG (Table 6.2). However, there appears to be inconsistent evidence between 25OHD concentration and MetS components. A large British study (n=7,198) of middle-aged (45 years) Caucasian adults found an inverse association between 25OHD concentration and high WC, high BP, high HbA1c and high TG but not with low HDL-C (Hypponen et al., 2008). We too found an association between 25OHD and elevated TG and FPG, however no associations with other MetS components. An American study (n=8,421) found an inverse association between 25OHD and high WC, TG and FPG (Ford, Ajani, McGuire, & Liu, 2005), however we found no association with WC. In an older cohort,

25OHD concentration was inversely associated with high WC, TG, FPG, and reduced HDL-C, however no association was seen with BP (Vitezova et al., 2015). A large prospective study from Australia (n=4,164) found an inverse association between 25OHD and the incidence of WC, TG, FPG, but not with HDL-C and BP (Gagnon et al., 2012). The variation in results amongst previous studies and our findings may be due to a few reasons, such as the use of different MetS criteria and cut-off points for 25OHD concentration. The majority of these associations were found in a middle aged or older population, however our study covered adults aged 18-75 years (Lee et al., 2009; Vitezova et al., 2015; Yin et al., 2012). Older populations may be at higher risk of vitamin D deficiency, due to certain physiological impairments that occur with age, thus the results from older adults may not be applicable to the general population (Timpini, Pini, Tantucci, Cossi, & Grassi, 2011). Evidence drawn from specific cultural groups (Hypponen et al., 2008; Lee et al., 2009; Yin et al., 2012), genders (Lee et al., 2009) and the use of differing confounders may also partially explain the variation in results.

6.4.2 Previous studies of dietary Ca intake and MetS components

There are limited studies examining the association between Ca and MetS components. In a large cohort study (n=6,375) of adults aged ≥40 years, the highest quartile of Ca intake (median 530.8 mg/d) was inversely associated with high WC and high blood glucose in women, and hypertriglyceridemia in men (Shin et al., 2015). A second large study (n=10,066) in women found a lower prevalence of lower WC, high BP, and high HDL–C in the highest quintile of dietary Ca intake (Liu et al., 2005). However we found an association between 500 mg/d increments in dietary Ca intake and reduced odds of elevated DBP. We found no significant association between Ca intake and any other MetS components, after controlling for demographic, physical, dietary factors and MetS components. Results may vary due to the investigation of one gender (Liu et al., 2005), older age group (≥40 years vs. ≥45 years vs. 30-65 years) (Drouillet et al., 2007; Liu et al., 2005; Shin et al., 2015) vs. a broad age range of 18-75 years in our study, and the adjustment for different confounders. In addition, these

studies did not mutually adjust for MetS components in their analysis, thus the associations between MetS components and dietary Ca intake may be confounded by the presence of other MetS components. The association between dietary Ca intake and MetS components has not been well investigated, and requires further research.

6.4.3 The combined effect of 25OHD concentration and dietary Ca intake on MetS components

Calcium intake and vitamin D play an inter-dependent role in bone metabolism and skeletal health. It was therefore appropriate to examine their combined role in extra-skeletal health. The combined effect of 25OHD concentration and Ca intake tertiles lowered the odds of having reduced HDL-C, and elevated TG, and the reduction in AOR of having these components was greater than that obtained from the independent effects of 25OHD concentration or dietary Ca intake.

Independently every 10 nmol/L increment in 25OHD reduced odds of elevated TG by 21%. However the combination of medium 25OHD tertile and low, and medium Ca intake, appeared to reduce the odds of elevated TG further by 43% and 41%, respectively (Figure 6.2). There also appeared to be a dose-response effect of Ca intake at low 25OHD concentration, though the combinations were not significant. The odds of having elevated TG were approximately halved between medium 25OHD to high 25OHD.

Increments in 25OHD and Ca intake did not significantly reduce the odds of elevated HDL-C. However, in combination, there appeared to be a strong effect of Ca at low 25OHD on reduced HDL-C. The combination of low 25OHD and medium and high Ca intake lowered the odds of having reduced HDL-C by 47% and 64% respectively (Figure 6.1). The combination of 25OHD concentration and Ca intake appeared to further reduce the odds of having reduced HDL-C than when 25OHD concentration was taken into account alone. However, at high 25OHD concentration, the effects of Ca appear to be blunted.

Independently 500 mg/d increment in dietary Ca intake appeared to reduce the odds of elevated DBP by 22%, with a marginal association between 25OHD concentration and elevated DBP. The combined effect of high 25OHD and medium Ca tertiles revealed 43% lower odds of having elevated DBP, which was marginally significant (p=0.080) (Table 6.4).

We are unaware of other studies that have investigated the combined effect of 25OHD concentration and Ca intake on MetS components. From our results it appears that there may be a combined effect of Ca to 25OHD in reducing the odds of having reduced HDL-C, and elevated TG. There appeared to be a strong effect of Ca at low 25OHD on reduced HDL-C. Whereas there appeared to be a stronger effected of Ca at high 25OHD on elevated TG. The potential mechanisms are explored below.

6.4.4 Potential mechanisms

6.4.4.1 25OHD concentration and MetS components

The pathophysiological mechanisms linking vitamin D to the MetS components, is not yet confirmed. One potential mechanism may be via the renin angiotensin system (RAS). The RAS is the key regulatory system of cardiovascular function, particularly for BP (Schmieder et al., 2007), however it has regained attention for its potential action in aspects of MetS (de Kloet, Krause, & Woods, 2010). Vitamin D may play a role in modulating BP through regulating the RAS, and renin production (the enzyme involved in maintaining BP) (Li et al., 2004). Vitamin D deficiency has been found to increase RAS activity (Dluhy & Williams, 2004; Vaidya et al., 2011) and stimulate renin synthesis. Increased RAS and renin activity can result in hypertension and insulin resistance (IR), which are characteristics of MetS (Engeli et al., 2003; Li et al., 2004). Secondly, IR may also be related to RAS whereby vitamin D deficiency may increase renin-angiotensin II expression, which may induce IR (Leiter & Lewanczuk, 2005; Rammos, Tseke, & Ziakka, 2008; Wei et al., 2008). In addition, the vitamin D pathway is involved in insulin secretion through the regulation of intracellular Ca^{2+} concentration (Sooy et al., 1999), and lower levels of vitamin D are linked with increased IR

(Chiu et al., 2004; Chonchol & Scragg, 2007). Thirdly, 25OHD modulates enzyme activity directly related to lipoprotein lipase (Querfeld et al., 1999). Vitamin D and its metabolites may upregulate lipoprotein lipase and in turn increase HDL-C and reduce TG (Querfeld et al., 1999). Fourthly, vitamin D may reduce inflammation and as a result reduce IR leading to improved lipid metabolism (Chagas, Borges, Martini, & Rogero, 2012; Hewison, 2012). Overall, vitamin D deficiency may increase RAS activity which is linked with the pathogenesis of hypertension, reduced insulin secretion, and IR (Vaidya & Williams, 2012).

6.4.4.2 Dietary Ca intake and MetS components

The positive effect of Ca on the MetS components may occur through different pathways. Those with lower Ca intakes (<600 mg/d) tend to have higher body weight, BMI, % fat mass, fat mass, WC and abdominal adiposity (Jacqmain et al., 2003). Dietary Ca intake may increase fat oxidation which may in turn lower lipid levels (Soares et al., 2011a; Soares et al., 2012). Zemel (1998) suggested that low Ca intake may affect hormones associated with bone growth. An increase in parathyroid hormone (PTH) and $1,25OH_2D$ may result in a rise in intracellular Ca^{2+} concentrations influencing lipogenesis (Zemel, 1998; Zemel et al., 2000). Higher intakes of Ca of ~1200 mg from dairy sources, may increase faecal fat excretion by ~5 g/d (Christensen et al., 2009). A meta-analysis of randomized controlled trials (RCTs) found that Ca supplementation of ~1000 mg/d reduced SBP and DBP, however those with lower Ca intake had a greater decrease in BP (van Mierlo et al., 2006). Interestingly, those who were Ca deficient and receiving Ca supplements tended to have a stronger effect on BP, than those with higher habitual intakes.

From our results, it appears that the additive effect of 25OHD and Ca is more beneficial than 25OHD alone, in reducing the odds of having reduced HDL-C, and elevated TG. There appears to be a dose-response effect of Ca at low 25OHD on reduced HDL-C, but at higher 25OHD, the effects of Ca were blunted (Figure 6.1). However, high 25OHD appeared to lower the odds of elevated TG, irrespective of Ca intake (Figure 6.2). This indicates that there a

multitude of mechanisms at play for each component constituting a complex relationship between 25OHD, Ca intake and MetS components.

6.4.5 Strengths

There are several strengths to our study. We draw our findings from a large population based sample of adults aged 18-75 years. We adjusted for a range of demographic and dietary variables as compared to other studies (Drouillet et al., 2007; Hypponen et al., 2008; Lee et al., 2009; Liu et al., 2005; Shin et al., 2015; Vitezova et al., 2015; Yin et al., 2012). In our study, 25OHD concentration was measured at one laboratory, rather than multiple laboratories which may limit variability in 25OHD results (Black et al., 2015). In contrast to other studies (Drouillet et al., 2007; Liu et al., 2009; Shin et al., 2015) we adjusted for the effect of other MetS components in the final model. This allowed us to see if the association between 25OHD and Ca tertiles and MetS components were independent from the rest of the components.

6.4.6 Limitations

The cross-sectional study design is good to show an association between two variables, however causality and National Health and Medical Research Council level 2 evidence can be obtained from longitudinal cohort studies (National Health and Medical Research Council, 1999). Supplement usage was not collected in the VHM survey, thus teasing out the role of Ca from food vs. a pharmacological source would be important to public health recommendations. Furthermore, Ca consumed as part of a food or food matrix, has varying absorbability and hence metabolic effects. Future studies may wish to explore the differences between dietary Ca vs. Ca as part of a food matrix for any metabolic differences (Zemel, 2009). The database lacked information on sun exposure and vitamin D supplement use thus the contribution of each could not be investigated. The lack of data on markers of IR, such as HOMA-IR, limits our understanding of the effects of 25OHD, and Ca intake on glucose homeostasis (Singh & Saxena, 2010). However unlike some studies we have controlled for other individual components of MetS which would be strongly related to IR. PTH is crucial for 25OHD

concentration and Ca metabolism, however this was not measured in our participants. A raised PTH can increase MetS components (Ahlström et al., 2009; Reis et al., 2008) and controlling for such a confounder would be important.

6.5 Conclusion

Our findings have indicated that increments of 10 nmol/L of 25OHD concentration may lower the likelihood of having elevated TG, and FPG, and 500 mg/d increments in dietary Ca intake may reduce the odds of elevated DBP. The combination of 25OHD and Ca tertiles appears to further reduce the odds of reduced HDL-C, and elevated TG. This warrants further investigation of the optimal level of the combination of 25OHD and dietary Ca intake in reducing the risk of MetS components. Future prospective longitudinal studies and high quality RCTs of vitamin D and/or calcium supplementation are needed to evaluate whether there is a causal relationship between 25OHD concentration, dietary Ca intake and individual MetS components.

Chapter 7 Vitamin D status is inversely associated with markers of risk for type 2 diabetes: a population based study in Victoria, Australia

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Objective addressed

Objective 5: To investigate the association between vitamin D status and the risk of T2DM.

7.1 Introduction

Vitamin D status, as judged from circulating concentrations of 25OHD, is a worldwide concern. In many countries across all continents, approximately 50% of those populations have an inadequate 25OHD status (<50 nmol/L) (Lips, 2010). Countries like India and China have some of the highest rates of vitamin D deficiency (<25 nmol/L) (Lips, 2010). The 25OHD status of Australians is also surprisingly low for a country blessed with abundant sunshine. Current estimates indicate that ~31% of the population have inadequate 25OHD levels (Daly et al., 2012), with a higher prevalence in older Australians (Nowson et al., 2012). The prevalence of T2DM has also risen tremendously in the last 10 years, with projections that countries like India and China will have the highest numbers by 2030 at 79.4 and 42.3 million respectively (Wild, Roglic, Green, Sicree, & King, 2004).

There is an ongoing interest in the extra-skeletal effects of vitamin D including its potential to blunt the risk of developing T2DM. Positive outcomes would present a tangible public health solution, if causality is accepted. Such a relationship was first suggested in 1967 by Milner and Hales, who found that insulin secretion in rabbits was dependent on calcium and magnesium, which are tightly regulated by vitamin D (Milner & Hales, 1967). Accumulating evidence has indicated that higher 25OHD status may have several anti-diabetic effects, including improvement in insulin sensitivity, stabilising HbA1c levels (Nagpal, Pande, & Bhartia, 2009), and improving beta cell function (Mitri et al., 2011a), whereas low 25OHD status may increase risk of T2DM (Gagnon et al., 2011). Thus, in the current environment of increasing rates of T2DM (International Diabetes Federation, 2013), their close parallelism with insufficient levels of 25OHD deserves investigation in population based studies. There are several lifestyle factors that modulate the risk of T2DM, including dietary components and patterns (Cespedes et al., 2016; Ley, Hamdy, Mohan, & Hu, 2014), physical activity, and smoking (Shamshirgaran, Jorm, Bambrick, & Hennessy, 2013). The risk of developing T2DM over 20 years appears to increase with the accumulation of metabolic syndrome (MetS)

components. The risk of T2DM increased by: 11.9% in those with zero Mets components, 31.2% in those with three MetS components and 40.8% in those with four or five MetS components (Wannamethee et al., 2005). Though the presence of MetS components increases the risk of T2DM, glucose is the most strongly correlated factor in predicting the development of diabetes in the future (Nichols & Moler, 2010). In a study of more than 58, 000 adults, as the number of components increased, so did the incidence of diabetes (Nichols & Moler, 2010). However, some gaps may exist with one study in Hispanic Americans finding that impaired glucose tolerance had a greater predictive power than the individual MetS components (Lorenzo, Okoloise, Williams, Stern, & Haffner, 2003). Thus, the presence of MetS is another major risk predictor of increased T2DM (Bonora et al., 2004).

The aim of this study was to investigate the association of 25OHD, and the risk of T2DM. We (Pannu et al., 2016c) as well as others (Chon et al., 2014; Ford et al., 2005; Gagnon et al., 2012; Vitezova et al., 2015), have shown that higher 25OHD status significantly reduced the risk of MetS and its components. Hence it was essential to adjust for several lifestyle factors and components of MetS, other than glucose, in order to correctly identify any independent association between 25OHD and risk of T2DM. One other study (Gagnon et al., 2011) has also investigated the association between 25OHD and T2DM and adjusted for three out of the four MetS components. Thus our study appears to be one of the first to adjust for all MetS components. Impaired FPG and HbA1c levels are now recommended as key determinants of early risk of T2DM (American Diabetes Association, 2010). While high FPG is an immediate indicator of poor glucose homeostasis, HbA1c is a better indicator of longer term control of blood glucose, and recommended cut-offs for both these biomarkers are used to diagnose T2DM (American Diabetes Association, 2010). The underlying hypothesis of the present investigation was that increases in 25OHD would reduce the odds ratio of a high FPG and a high HbA1c after adjustment for socio-demographic, dietary and biomedical confounders.

7.2 Materials and Methods

7.2.1 Sample

The VHM survey was a state-wide cross-sectional population based study (Department of Health, 2012a) conducted in Victoria, Australia. Victoria lies in the south-east of Australia, and has a latitude of 37º47'S and longitude of 144º58'E. Data was collected between May 2009 and April 2010 including: physical information, dietary behaviour information and biomedical information. The physical and biomedical information of participants were collected by trained staff at four training sites. The VHM employed a stratified cluster sample selection method of Census Collection Districts within eight Department of Health regions in Victoria. Data were collected on 3,653 adults aged 18-75 years. From this sample, we excluded participants with: missing HbA1c and FPG data (n=31), those with HbA1c ≥6.5% (n=39) and FPG >7 mmol/L (n=16) as they were classified as having T2DM as per the ADA cut-offs (American Diabetes Association, 2010), those with T2DM (n=140), those with T1DM (n=9), and those on diabetic medications (n=25). A total of 3,393 subjects were included in this analysis. Further details on physical, dietary, and biomedical data collection and analysis have been previously described (Department of Health, 2012a, 2012b; Pannu et al., 2016c).

7.2.2 Biomedical measurements

Participants attended a testing site after an overnight fast of at least ten hours. Blood samples were collected by venepuncture, and were subsequently transported to a central laboratory in Melbourne, Australia. Bloods were analysed for: FPG, HbA1c, 25OHD, HDL-C, and TG. The components in the blood were measured as follows: FPG using the hexokinase method, HbA1c using immunosassay (Roche Integra chemistry analyser), 25OHD concentration were measured based on the DiaSorin Corporation Liaison® 25OHD total assay HDL-C using elimination/catalase method; and TG using GPO Trinder reagent set with serum

blank. The BP of participants was measured by survey staff using an automated BP monitor, which was calibrated regularly (Department of Health, 2012a).

7.2.3 Physical measurements

The anthropometric measurements were made at the testing sites by trained staff, and included height, weight and WC. Height was measured without shoes using a stadiometer. Weight was measured without shoes and light clothing, using a digital weighting scale. Waist circumference was measured using a steel measuring tape. Body mass index was calculated from the weight and height measurements (Department of Health, 2012a).

7.2.4 Dietary and physical activity measurements

Dietary information was collected by multiple-pass 24 hour dietary recall using computer assisted telephone interviews. Dietary recall interviews were conducted by dietitians from the Department of Nutrition and Dietetics at Monash University in Melbourne, Australia. The FoodWorks® nutrition software (FoodWorks® Interview) were used for conducting the dietary recalls. Based on the three dietary recalls, the mean intake for each nutrient was calculated and used in the analysis (Department of Health, 2012a). Further information on the assessment of dietary intake data can be found in our previous publications (Department of Health, 2012a; Pannu, Calton, & Soares, 2016a). Physical activity information was collected via interviews with the participant. The time spent in physical activity was calculated based on the sum of the time spent walking or performing moderate activity plus double the time spent in vigorous activity (to indicate its greater intensity) (Armstrong et al., 2000; Department of Health, 2012a).

7.2.5 FPG and HbA1c

Fasting plasma glucose (FPG) and HbA1c were the two dependent variables. The ADA cut-offs were used to identify those who were at a risk of T2DM based on FPG and HbA1c levels (American Diabetes Association, 2010). A binary variable was used to

categorise subjects as being at low or high risk for T2DM: FPG <5.6 mmo/L (low risk, normal, coded '0'), vs. 5.6-6.9 mmol/L (high risk, coded '1'), and HbA1c <5.7% (low risk, normal, coded '0') vs. 5.7-6.4% (high risk, coded '1').

7.2.6 25OHD concentration

25-hydroxyvitamin D concentration was the primary independent variable of interest. 25OHD concentration was categorised as tertiles: low 25OHD (median 10-44 nmol/l), medium 25OHD (median 45-65 nmol/L) and high 25OHD (median 65-204 nmol/L).

7.3 Statistical analysis

7.3.1 Socio-demographic factors

In our analysis we considered a number of confounders, based on our (Markwick et al., 2015) and others experience in the area (Krishnan, Rosenberg, & Palmer, 2009; Shamshirgaran et al., 2013). We adjusted for the following socio-demographic factors: age, gender, county of birth, Index of Relative Socio-economic Disadvantage (IRSED), physical activity, smoking status, and season. Age were entered as continuous variables. Country of birth was categorised as those born in Australia or overseas. The socio-economic indicator used was the IRSED, which is an index based on the social and economic conditions of individuals within an area (Australian Bureau of Statistics, 2011). Subjects were categorised into IRSED quartiles: quartile 1 (most disadvantaged), quartile 2 (disadvantaged), quartile 3 (less disadvantaged), and quartile 4 (least disadvantaged). Physical activity levels were classified into three categories: sufficient activity (≥150 minutes/week), insufficient activity (1-149 minutes/week), and inactive (0 minutes/week). There is a known seasonal variation to FPG and HbA1c, so we had to adjust for season (Gikas et al., 2009; Tseng et al., 2005). Season of biomedical assessment refers to the season of the year that the participant attended the testing site, and had their bloods collected for assessment of 25OHD status. Season of

biomedical assessment were grouped as summer, autumn, winter, and spring. Smoking status were categorised into three categories: current smoker, ex-smoker, and non-smoker.

7.3.2 Dietary factors

Based on previous research (Dong, Xun, He, & Qin, 2011; Mitri, Muraru, & Pittas, 2011b; Yang et al., 2014) dietary factors included in the analyses were: dietary fibre, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake and under/over reporting of energy intake. Self-reported energy intake may result in the under or over reporting of true energy intake (EI), and this may confound the estimation of any diet and disease related outcomes (Goldberg & Black, 1998). We predicted basal metabolic rate (BMR) using the Henry/Oxford equations based on a range of ages (18-30, 30-60, 60-70, and 70 and above years), gender and body weight (Henry, 2007). Rather than use the Goldberg cut-offs to identify under-reporters and over-reporters (Goldberg et al., 1991), we calculated the ratio of energy intake to BMR (EI:BMR) and treated it as a confounder. Dietary fibre, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake, and under/over reporting of energy intake were all entered as continuous variables.

7.3.3 Biomedical factors

Biomedical factors included in the analyses were: MetS components including WC, HDL-C, TG, BP, as well as BMI, and haemoglobin levels (HbA1c model only). Haemoglobin levels were adjusted for in the HbA1c model only, as recent findings have indicated that haemoglobin levels may increase HbA1c levels (Attard et al., 2015; Kim, Bullard, Herman, & Beckles, 2010). BMI and haemoglobin levels were entered as a continuous variable. Those with MetS tend to be at higher risk of developing T2DM, thus we adjusted for MetS components in the HbA1c and FPG model (Grundy, 2012). MetS components were each classified as binary variables, as defined by the joint interim statement (Alberti et al., 2009). Waist circumference were: normal WC (<94cm for males or if Aboriginal or Torres Strait Islander (ATSI), Asian or South American <90cm; <80cm for females), or elevated WC (≥

94cm for males or if ATSI, Asian or South American ≥90cm; ≥80cm for females). HDL-C were: normal HDL (≥1.0 mmol/L for males; ≥1.3 mmol/L for females), or low HDL (<1,0 mmol/l for males; <1.3 mmol/L for females). TG were: normal TG (<1.7 mmol/l), or hypertriglyceridaemia (≥1.7 mmol/l). BP were: normal BP (<130/85 mmHg and no anti-hypertensive medications), or high BP (≥130/85 mmHG or on anti-hypertensive medications) (Alberti et al., 2009).

7.3.4 Statistical analysis

The statistical analysis was conducted in the following two stages:

Step 1: Descriptive statistics for HbA1c and FPG were obtained and normality was assessed for variables of interest (natural logarithm transformation was applied if variable was skewed). Differences between groups were then examined by Independent samples *t* test and χ^2 test.

Step 2: Multiple logistic regression analyses were employed to obtain AOR and 95% CI for the associations between serum 25OHD and having higher HbA1c and FPG, respectively. Three categories of variables were used in the regression models including: *socio-demographic variables* (age, sex, country of birth, IRSED, physical activity, smoking status, and season), *dietary factors* (dietary fibre, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake, and under/over reporting of energy intake) and *biomedical variables* (MetS components: WC (ethnic specific cut-offs), HDL-C (normal/low), TG (normal/high), BP (normal/high), BMI and haemoglobin levels for the HbA1c model only). All these variables have a known association with FPG and HbA1c and have been previously reported in the literature.

The primary research variable of interest, serum 25OHD, was entered into the multiple regression model as 1) a continuous variable (10 nmol/L increments) and also as 2) a

categorical variable based on 25OHD tertiles. For both continuous and categorical 25OHD, the multiple regression model was initially adjusted for the socio-demographic variables in Model 1; secondly both socio-demographic variables, dietary factors and haemoglobin levels (HbA1c model only) in Model 2; and finally all socio-demographic, dietary factors and biomedical variables altogether in Model 3. The IBM SPSS Statistics for Windows, Version 21.0, was used for the statistical analyses. The VHM survey employed the use of the multistage stratified cluster-sampling procedure. In order to adjust for the unequal selection probability due to this sampling method, complex samples analysis was used. Complex samples approach takes into account the complex survey sampling and selection probability used in the VHM survey. Variables (strata variable, weighting variable and clustering variable) describing the survey design in terms of stratification, clustering and multistage sampling were entered into the SPSS complex samples approach for generating sampling weights in estimation and standard errors. A two-tailed p value of less than 0.05 was accepted as statistical significance.

7.4 Results

An overview of socio-demographic, dietary and clinical characteristics of subjects based on FPG and HbA1c levels are shown in Table 7.1. The prevalence of those with normal FPG (<5.6 mmol/L) was 84% and for high FPG (5.6-6.9 mmol/L) was 16%. 61% of the population had normal HbA1c (<5.6%), while 39% had high HbA1c (5.7-6.4%).

Table 7.1 Socio-demographic and clinical characteristics of participants by FPG and HbA1c.

	FPG (<5.6 mmol/L) n=2866 (84%)	FPG (5.6-6.9 mmol/L) n=527 (16%)	P value	HbA1c (<5.7%) n=2068 (61%)	HbA1c (5.7-6.4%) n=1325 (39%)	P value
	Mean (SE) *or* N (SE) %	Mean (SE) *or* N (SE) %		Mean (SE) *or* N (SE) %	Mean (SE) *or* N (SE) %	
Age (y)	42 (0.8)	52 (1.4)	<0.001	40 (0.9)	52 (0.9)	<0.001
BMI (kg/m^2)	26.6 (0.2)	29.2 (0.4)	<0.001	26.2 (0.2)	28.8 (0.2)	<0.001
Gender			<0.001			0.494
Males	1285 (1.5) 81%	299 (1.5) 19%		1112 (2.5) 70%	472 (2.5) 30%	
Females	1663 (1.0) 91%	162 (1.0) 9%		1257 (2.4) 69%	577 (2.4) 31%	
Country of birth			0.031			<0.001
Born in Australia	2264 (0.7) 88%	320 (0.7) 12%		1868 (2.1) 72%	716 (2.1) 28%	
Born overseas	674 (1.9) 83%	134 (1.9) 17%		505 (3.6) 62%	303 (3.6) 38%	
IRSED			0.020			0.216
Most disadvantaged	705 (1.7) 83%	142 (1.7) 17%		545 (5.1) 64%	302 (5.1) 36%	
Disadvantaged	732 (1.0) 87%	108 (1.0) 13%		570 (3.8) 68%	270 (3.8) 32%	
Less disadvantaged	765 (1.3) 89%	98 (1.3) 11%		639 (4.1) 74%	224 (4.1) 26%	
Least disadvantaged	737 (1.2) 87%	106 (1.2) 13%		631 (3.3) 75%	212 (3.3) 25%	
Smoking status			<0.001			0.002
Current smoker	441 (2.2) 84%	86 (2.2) 16%		366 (3.7) 69%	161 (3.7) 31%	
Ex-smoker	733 (2.0) 80%	179 (2.0) 20%		575 (3.2) 63%	337 (3.2) 37%	
Non-smoker	1756 (0.9) 90%	195 (0.9) 10%		1418 (2.1) 73%	533 (2.1) 27%	
25OHD concentration						
Serum 25OHD (nmol/L)	56.7 (2.0)	52.1 (2.5)	0.081	57.2 (2.2)	53.6 (1.9)	0.208
25OHD tertiles			0.045			0.135
Low 25OHD (33 nmol/L)	933 (1.5) 84%	180 (1.5) 16%		745 (2.9) 67%	359 (2.9) 33%	
Medium 25OHD (54 nmol/L)	992 (1.3) 85%	168 (1.3) 15%		798 (3.5) 69%	32 (3.5) 31%	
High 25OHD (77 nmol/L)	1013 (1.5) 90%	116 (1.5) 10%		829 (2.4) 73%	300 (2.4) 27%	
Dietary variable						
Energy (kJ/d)	9687.4 (116.5)	9784.9 (164.9)	0.653	9904.4 (147.8)	9236.4 (145.6)	<0.001
Biomedical factors						

	FPG (<5.6 mmol/L) n=2866 (84%)	FPG (5.6-6.9 mmol/L) n=527 (16%)	P value	HbA1c (<5.7%) n=2068 (61%)	HbA1c (5.7-6.4%) n=1325 (39%)	P value
Waist circumference (cm)	88.0 (0.7)	96.9 (1.1)	<0.001	86.9 (0.7)	94.7 (0.9)	<0.001
Triglycerides (mmol/L)	1.2 (0.03)	1.5 (0.04)	<0.001	1.1 (0.03)	1.5 (0.04)	<0.001
HDL-C (mmol/L)	1.5 (0.02)	1.4 (0.03)	<0.001	1.5 (0.02)	1.4 (0.02)	<0.001
Systolic blood pressure (mmHg)	123 (0.6)	133 (1.1)	<0.001	123 (0.7)	128 (0.6)	<0.001
Diastolic blood pressure (mmHg)	73 (0.5)	77 (0.7)	<0.001	72 (0.5)	76 (0.5)	<0.001
Haemoglobin levels (g/L)	142.9 (0.4)	148.2 (1.1)	<0.001	144.2 (0.4)	142.4 (0.6)	<0.001

Footnotes: Data are presented as mean estimate (weighted) (%) for categorical variables, and mean estimate (weighted) and (SE) for normal continuous variables. Difference in the continuous and categorical variables between groups were assessed by independent samples t-test (natural logarithm transformation was used if the variable was not normal) and Chi-square test (association between FPG or HbA1c and categorical variables, with an emphasis on which category were more likely to have high FPG, or high HbA1c), respectively.

Legend: d, day; SE, standard error; min, minutes; wk, week.

7.4.1 Association between serum 25OHD and FPG

When the serum 25OHD was entered as a continuous covariate to the multiple regression model, for every increment in serum 25OHD of 10 nmol/L, the odds of having higher FPG reduced by 9% (AOR 0.91, 95% CI 0.86, 0.97; p=0.002) after adjusting for socio-demographic variables, dietary factors, and biomedical variables in Model 3. For all models when serum 25OHD was entered as a categorical factor, compared with those people in the low 25OHD tertile, those with the high 25OHD tertile had a significantly reduced risk of higher FPG. More specifically, after adjustment for both socio-demographic, dietary factors and BMI in Model 2 the odds of having higher FPG reduced by 40% for those in high 25OHD tertile (AOR 0.60, 95% CI 0.43, 0.83; p=0.008) vs. low 25OHD tertile. After further adjustment for MetS components in Model 3, the AOR appeared relatively stable with a 39% reduced odds of higher FPG in the high 25OHD vs. low 25OHD tertile (AOR 0.61, 95% CI 0.44, 0.84; p=0.011) (Table 7.2).

Table 7.2 The association of serum 25OHD and FPG: crude and adjusted odds ratio and their 95% CI based on logisitic regression.

	Model 1		Model 2		Model 3	
	COR	**95% CI**	**AOR**	**95% CI**	**AOR**	**95% CI**
25OHD continuous (10 nmol/L)	0.93	0.87, 1.01	0.91	0.86, 0.97	0.91	0.86, 0.97
P value	0.054		0.003		0.002	
	Model 1		**Model 2**		**Model 3**	
25OHD tertiles	**COR**	**95% CI**	**AOR**	**95% CI**	**AOR**	**95% CI**
Low 25OHD (33 nmol/L) †	1.0		1.0		1.0	
Medium 25OHD (54 nmol/L) †	0.89	0.60, 1.31	0.87	0.57, 1.32	0.87	0.59, 1.29
High 25OHD (77 nmol/L) †	0.66*	0.46, 0.94	0.60*	0.43, 0.83	0.61*	0.44, 0.84
P value for trend	0.076		0.008		0.011	

Model 1: age, sex, country of birth, IRSED, physical activity, smoking status, season, BMI.

Model 2: Model 1 plus dietary fibre, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake, under/over reporting of energy intake.

Model 3: Model 2 plus WC, HDL-C, TG , BP ; all as categorical variables based on MetS cut-offs.

Legend: COR, crude odds ratio; AOR, adjusted odds ratio; 1.0, lowest 25OHD served as the reference group.

Footnotes: *, significant in comparison to reference group at 5% significance level; †, median of the tertile group.

7.4.2 Association between serum 25OHD and HbA1c

In Model 3 after adjustment for socio-demographic, dietary factors, and biomedical variables every 10 nmol/L increment in 25OHD also significantly reduced the odds of higher

HbA1c by 6% (AOR 0.94, 95% CI 0.90, 0.98; p=0.009). In Model 2, after adjustment for socio-demographic, dietary factors, BMI and haemoglobin levels, there was a significantly reduced odds of having higher HbA1c by 33% in those with high vs. low 25OHD tertile. After further adjustment for MetS components in Model 3, a 26% reduced odds of higher HbA1c (AOR 0.74, 95% CI 0.58, 0.93; p=0.041) were found in the high 25OHD tertile group, compared to the low 25OHD tertile group (Table 7.3).

Table 7.3 The association of serum 25OHD and HbA1c: crude and adjusted odds ratio and their 95% CI based on logistic regression.

		Model 1		Model 2		Model 3
	COR	**95% CI**	**AOR**	**95% CI**	**AOR**	**95% CI**
25OHD continuous (10 nmol/L)	0.93	0.88, 0.97	0.93	0.88, 0.97	0.94	0.90, 0.98
P value	0.002		0.002		0.009	
	Model 1		**Model 2**		**Model 3**	
25OHD tertiles	**COR**	**95% CI**	**AOR**	**95% CI**	**AOR**	**95% CI**
Low 25OHD (33 nmol/L) †	1.0		1.0		1.0	
Medium 25OHD (54 nmol/L) †	0.78	0.56, 1.09	0.79	0.56, 1.11	0.83	0.58, 1.17
High 25OHD (77 nmol/L) †	0.68*	0.54, 0.86	0.67*	0.53, 0.85	0.74*	0.58, 0.93
P value for trend	0.007		0.005		0.041	

Model 1: age, sex, country of birth, IRSED, physical activity, smoking status, season, BMI.

Model 2: Model 1 plus dietary fibre, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake, under/over reporting of energy intake, haemoglobin levels.

Model 3: Model 2 plus WC, HDL-C, TG, BP; all as categorical variables based on MetS cut-offs.

Legend: COR, crude odds ratio; AOR, adjusted odds ratio; 1.0, lowest 25OHD served as the reference group.

Footnotes: *, significant in comparison to reference group at 5% significance level; †, median for the tertile group.

7.5 Discussion

The results from this population based study of adults from Victoria, Australia indicate that higher 25OHD levels may ~~be~~ significantly ~~related to~~ lower the risk of higher FPG and HbA1c levels. This significant inverse association persisted after the adjustment for a number of socio-demographic, dietary, and biomedical variables and MetS components. We found that those in the high 25OHD tertile had a 39% reduced risk of higher FPG and 26% reduced risk of higher HbA1c, when compared to the low 25OHD tertile. This was independent of MetS components, and haemoglobin levels in the HbA1c model, which have been found to confound the association between 25OHD and T2DM. Three recent meta-analysis (Jamka et al., 2015; Krul-Poel, Ter Wee, Lips, & Simsek, 2017; Poolsup et al., 2016) found no significant effect of vitamin D supplementation on IR (Jamka et al., 2015; Poolsup et al., 2016), FPG (Jamka et al., 2015; Krul-Poel et al., 2017) or HbA1c (Krul-Poel et al., 2017). Though, one of these studies found a beneficial effect of vitamin D supplementation on FPG and HbA1c (Poolsup et al., 2016). Our recent review summarised that although there were inverse associations between 25OHD and IR, the systematic reviews and meta-analysis in this review did not favour a casual role (Soares, Pannu, Calton, Reid, & Hills, 2017). Our findings are in line with previous observational studies that found an inverse association between 25OHD status and high FPG levels (Forouhi et al., 2008; Gagnon et al., 2012) and high HbA1c levels (Hutchinson, Figenschau, Njolstad, Schirmer, & Jorde, 2011; Hypponen & Power, 2006; Kositsawat et al., 2010; Manickam, Neagu, Kukreja, & Barengolts, 2013; Need, O'Loughlin, Horowitz, & Nordin, 2005; Zoppini et al., 2013). However, these studies adjusted for fewer variables, with smaller sample sizes than our study and were not population based (Forouhi et al., 2008; Manickam et al., 2013; Need et al., 2005; Zoppini et al., 2013). The differences in findings between both interventional and observational based studies indicates a need for good quality RCTs.

A number of inter-related factors contribute to the pathogenesis of T2DM, including the presence of MetS components. One prospective study found that those with IR and high FPG (5.6-6.9 mmol/L) had double the risk of worsened cardio-metabolic profile after nine years (Salazar et al., 2016). Obesity, dyslipidemia and hypertension are MetS components which are often found in those with pre-diabetes (Ferrannini, Gastaldelli, & Iozzo, 2011). We adjusted for these variables in our analysis and found that the association between 25OHD and risk of T2DM still existed, irrespective of MetS. Other studies investigating 25OHD and risk of T2DM have adjusted for either none (Hypponen & Power, 2006; Kositsawat et al., 2010; Manickam et al., 2013), or one MetS components (Hutchinson et al., 2011; Mattila et al., 2007; Pittas et al., 2006), with one study accounting for all of the MetS components (Gagnon et al., 2011).

7.5.1 Potential mechanisms

The beneficial effect of 25OHD in reducing risk of T2DM is likely due to its effect on insulin action. The expression of the VDR in pancreatic beta cells indicates the importance of vitamin D in beta cell function (Cavalier et al., 2011; Norman, 1990) and insulin secretion (Milner & Hales, 1967). During times of vitamin D deficiency, beta cell function is blunted and insulin secretion is diminished (Boucher, 1998). Vitamin D may also indirectly influence insulin action via a calcium mediated effect. Vitamin D tightly regulates calcium homeostasis, whereby intracellular calcium levels are required to ensure effective action of insulin within different tissues (Cavalier et al., 2011). Vitamin D is also involved in the regulation of the renin angiotensin system (Al Mheid et al., 2013), endothelial vasodilation and lipid levels (Dalan, Liew, Tan, Chew, & Leow, 2014) which are mechanisms relating to the MetS components. T2DM and MetS are inter-twined, wherein IR appears to be a key player in the development of both conditions (Ferrannini, Haffner, Mitchell, & Stern, 1991; Reaven, 1988b). Though there are commonalties in the mechanisms underlying both conditions, our study found that even on adjustment for MetS components, the association between the high

25OHD tertile and lower odds of higher FPG and HbA1c levels persisted. This may potentially suggest that vitamin D has a beneficial role in T2DM, independent of MetS. However, the cross-sectional nature of this study does not provide further insight into this observation.

Low grade chronic inflammation is a hallmark of many chronic diseases and may precede T2DM (Pickup, 2004) possibly via initiation of IR (Keane, Cruzat, Carlessi, de Bittencourt, & Newsholme, 2015). Vitamin D has a role in immunity, and cellular studies show consistent reduction of inflammatory markers following cholecalciferol supplementation (Calton et al., 2015a). Adequate circulating 25OHD levels are required to obtain optimal anti-inflammatory responses in the body (Zhang et al., 2012), especially in those tissues like immune cells, where the enzymes for conversion of 25OHD to $1,25OH_2D$ are present. However, the optimum 25OHD levels for modulating inflammation responses are yet to be determined (Bikle, 2010). There is plausible but not confirmatory evidence to suggest the value is around 75-80 nmol/L (Heaney, 2008; Vieth, 2011); a point where maximum suppression of parathyroid hormone is also expected (Durazo-Arvizu et al., 2010). In support, a recent review (Pannu et al., 2016a), and emerging RCTs (Rosenblum, Castro, Moore, & Kaplan, 2012; von Hurst et al., 2010) found beneficial effects of vitamin D supplementation on IR and fat loss in those individuals who reached this value over the period of the trial (Rosenblum et al., 2012; von Hurst et al., 2010). In the present study those in the highest 25OHD tertile had a median of 77 nmol/L, where reduced risk of higher FPG and HbA1c was observed.

7.5.2 Limitations

The cross-sectional design does not afford causality of association, though we have controlled for several known confounders. The VHM did not collect information on supplement use, so we cannot separate the potential effects of vitamin D supplement and increased sun exposure on the higher 25OHD levels. Family history of T2DM may increase the risk of development of the disease (InterAct Consortium et al., 2013), but unfortunately

such information was not collected as part of the survey thus is a potential confounder. Approximately >75% of our sample were born in Australia, and due to small sample sizes we were unable to examine ethnic effects. Thus as ethnicity were not collected, results cannot be applied to all ethnic groups.

Studies have indicated that 25OHD may vary due to the genetic variation of three polymorphisms in the vitamin D genes, including the vitamin D binding protein, VDR and the 25OHD activating enzyme (Boucher, 2012; Wang et al., 2010). The concentration of the vitamin D binding protein may vary between individuals (Fu et al., 2009; Lauridsen et al., 2005) as well as fluctuations in its binding affinity (Arnaud & Constans, 1993). There may also be a genetic association between VDR polymorphisms and T2DM. Recent evidence has found that the polymorphism of certain VDRs may increase susceptibility to T2DM (Li, Wu, Liu, & Yang, 2013) and certain MetS components (Martinez-Hernandez et al., 2015). Lastly, the conversion of 25OHD to its active form requires CYP27B1, an enzyme that may be affected by genetic variation (Hypponen et al., 2009). These genetic variants were not studied as part of the VHM survey but certainly provide avenues for novel insights into the associations described here.

7.5.3 Strengths

This study used a representative sample of adult Australians from one state of the country. We adjusted for a wide range of socio-demographic, biomedical and dietary factors. Our study appears to be one of the few (Gagnon et al., 2011) which has accounted for all MetS components when investigating associations between 25OHD and T2DM. We adjusted for energy intake and the possible mis-reporting of energy intakes (Henry, 2007). That being said 24 hour recalls in this study were obtained from a five-pass method which is considered the gold standard for dietary information. Haemoglobin levels was adjusted for in the HbA1c analysis as evidence has indicated that anaemia may be associated with inaccurate HbA1c levels (Attard et al., 2015; Kim et al., 2010), whereas other studies in the area, did not adjust

for this factor (Gagnon et al., 2011; Hutchinson et al., 2011; Hypponen & Power, 2006; Kositsawat et al., 2010; Manickam et al., 2013; Mattila et al., 2007; Pittas et al., 2006).

7.6 Conclusion

Higher 25OHD status was associated with lower likelihood of higher FPG and higher HbA1c concentrations after accounting for socio-demographic, lifestyle variables and MetS components. Such outcomes could suggest a direct role for the vitamin in preventing T2DM.

Chapter 8 Summary and conclusions

The overall aim of the thesis was investigate the associations between vitamin D status and dietary calcium intake with the presence of MetS and its components, and the risk of T2DM by analysing the VHM survey dataset. Please refer to Apendix K for the papers that have been published during this PhD.

8.1.1 Objectives

Objective 1: To review the literature on vitamin D status, dietary calcium, and their association with MetS and T2DM.

Objective 2: To investigate the physical, demographic, and lifestyle determinants of vitamin D status in a population based sample of Australian adults aged 18-75 years

Objective 3: To verify the role of adiposity and weight loss on vitamin D status.

Objective 4a: To determine if vitamin D status, and/or dietary calcium intake are related to the presence of MetS.

Objective 4b: To determine whether vitamin D status, dietary calcium intake and their combination were associated with individual components of MetS.

Objective 5: To determine if vitamin D status is related to the risk of T2DM.

8.2 Key findings

As outlined below, we have contributed some interesting findings to the literature.

8.2.1 Determinants of vitamin D status

- We found that those who were: obese, were insufficiently physically active each week, were smokers, had a medium to high sitting time, living in metropolitan areas, and had

their biomedical examination in winter or spring had a reduced odds of medium and high 25OHD tertile. Those with medium dietary calcium intake had a higher odds of high 25OHD vs. low dietary calcium intake

For participants who were born in overseas, those with light brown skin colour, and fair skin colour had a higher odds of being in medium or high 25OHD tertile as compared with those with dark brown or black skin colour.

8.2.2 Weight loss on vitamin D status in obese subjects

In our meta-regression analysis we found a near significant association between weight loss and increase in 25OHD concentration. For every 10kg weight loss, there was an approximate increase of 6 nmol/L in 25OHD concentration. These results support the theory of volumetric dilution effect of vitamin D, however sequestration and the conversion to inactive metabolites can not be discounted.

These outcomes have been published as:

Pannu, P. K., Zhao, Y., & Soares, M. J. (2016). Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects: a systematic review and metaregression analysis. *Nutrition Research*, *36*(3), 201-213. doi:10.1016/j.nutres.2015.11.013.

8.2.3 Vitamin D status and dietary calcium intake and the presence of MetS and its components

We found that higher 25OHD concentration was associated with lowering the odds of MetS, reduced HDL-C, elevated TG, elevated DBP and elevated FPG. Dietary calcium had no effect on MetS and its components. Dietary calcium intake appeared to have a beneficial effect on MetS at low to medium 25OHD concentrations. The combination of 25OHD concentration and dietary calcium intake appeared to further reduce the odds of reduced HDL-C, elevated TG and elevated DBP.

The MetS outcomes have been published as:

Pannu, P. K., Zhao, Y., Soares, M. J., Piers, L. S., & Ansari, Z. (2016). The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor survey. *Public Health Nutrition*, 1-12. doi:10.1017/S1368980016001609.

The MetS components outcomes have been recently accepted for publication:

Pannu, P. K., Soares, M. J., Piers, L. S., Zhao, Y., & Ansari, Z. (2017). The association of vitamin D status and dietary calcium intake with individual components of the metabolic syndrome: a population based study in Victoria, Australia. Submitted to: *Cardiovascular Endocrinology*, Manuscript ID: CAEN-D-17-00009.

8.2.4 Vitamin D status and the risk of T2DM

We found that higher 25OHD concentration was associated with lowering the odds of higher FPG and higher HbA1c, thus lowering the potential risk of T2DM.

The T2DM outcomes have been published as:

Pannu P. K., Piers L. S., Soares, M. J., Zhao, Y., & Ansari, Z. (2017). Vitamin D status is inversely associated with markers of risk for type 2 diabetes: a population based study in Victoria, Australia. *PloS ONE, 12*(2), e0178825. doi: 10.1371/journal.pone.0178825.

8.3 Limitations

The cross-sectional nature of this study does not allow us to draw causal inferences. The response rate of the VHM survey was 38%, which was less than ideal. This creates a possibility of selection bias and non-reliability of survey estimates. To assess if any difference existed, the demographic information from those who refused to participate were compared to the Victorian Population Health Surveys in 2010 and 2011-12, which had a higher response rate

of 73% (Department of Health, 2012c) and 67% respectively (Department of Health, 2012c). There were minimal differences between the two, indicating that a low response rate had limited impact on the survey data.

Family history has been implicated as a risk factor in the development of MetS and T2DM, however this information was not collected. Recent evidence has indicated a role of genetic polymorphisms in potentially affect vitamin D status, as well as increasing the risk of T2DM and certain MetS components, however this information was not collected.

8.4 Future recommendations

- Collecting information on sun exposure and supplement use may help to tease out the effect of each on vitamin D status.
- The collection of genetic information may help identify which genes and the level of influence they have on vitamin D status, MetS and T2DM.
- The collection of information on inflammatory markers may assist in identifying its contribution in the development of MetS and T2DM.
- Prospective cohort studies to clarify and quantify the association between vitamin D status, calcium intake and MetS and T2DM.

8.5 Conclusion

This thesis explored the associations between vitamin D status and dietary calcium intake with the presence of MetS and its components and the risk of T2DM. This study found that of the determinants of low vitamin D status, sitting time was a unique factor. There appears to be both a volumetric dilution and a sequestration effect which may explain lower 25OHD in obese individuals. Vitamin D and dietary calcium may both have a role in the improvement of MetS and its components. Further the beneficial effects on the risk of T2DM were independent of MetS and its components. It is hoped that the results of this study may initiaite further exploration of the role of vitamin D and calcium in chronic disease.

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APPENDICES

Appendix A Calcium and vitamin D in obesity and related chronic disease

CHAPTER TWO

Calcium and Vitamin D in Obesity and Related Chronic Disease

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Contents

Abstract

There is a pandemic of lifestyle-related diseases. In both developed and lesser developed countries of the world, an inadequacy of calcium intake and low vitamin D status is common. In this chapter, we explore a mechanistic framework that links calcium and vitamin D status to chronic conditions including obesity, systemic inflammation, endothelial dysfunction, dyslipidemia and cardiovascular disease, and type 2 diabetes mellitus. We also update the available clinical evidence, mainly from randomized controlled trials, to provide a synthesis of evidence in favor or against these

[1] These authors have made equal contributions.

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hypotheses. There is consistent data to support calcium increasing whole body fat oxidation and increasing fecal fat excretion, while there is good cellular evidence for vitamin D reducing inflammation. Clinical trials support a marginal reduction in circulating lipids and some meta-analysis support an increase in insulin sensitivity following vitamin D. However, these mechanistic pathways and intermediate biomarkers of disease do not consistently transcribe into measurable health outcomes. Cementing the benefits of calcium and vitamin D for extraskeletal health needs a reexamination of the target 25(OH)D level to be achieved and the minimum duration of future trials.

1. INTRODUCTION

The worldwide prevalence of obesity has risen by approximately 28% in the last 33 years, and is now considered a pandemic (Ng et al., 2014). Rising levels of obesity were once seen as a sign of affluence (Haththotuwa, Wijeyaratne, & Senarath, 2013), however 62% of obese individuals live in developing countries (Ng et al., 2014). Excess weight gain increases the risk of chronic conditions such as metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and even some cancers. It is difficult to obtain global statistics on nutrient intakes, however one study investigated calcium intake within the United States, Russia, China, and India that together accounted for nearly half the world's population (Wang & Li, 2008). Calcium intake is closely tied to dairy product intake, and in all these countries there were upward trends in dairy production. However, in the United States, average calcium intake was below the recommended 1000 mg/d, with men's calcium intake at 962 mg and women's intake at 756 mg. In China, daily calcium intakes of 389 mg were well below the recommended 1000 mg, with less than 10% consuming the recommended amount. In most non-Western countries dietary intake is predominantly plant based, which is a poorer source of calcium. Nevertheless, even in the United States only 14% of the population consume more than the recommended three servings of dairy a day (Wang & Li, 2008).

Vitamin D deficiency is also a global health issue. Vitamin D status varies with skin type, latitude, foods fortified with vitamin D, use of supplements, sunshine exposure, and habitual clothing style. Adults who are at higher risk of vitamin D deficiency and insufficiency include pregnant women, elderly, and immigrants (Lips, 2010). The serum 25-hydroxyvitamin D (25(OH)D) concentration of <50 nmol/L as a cutoff for determining insufficiency is still debated, as it may not optimize the required levels for extraskeletal health

(Bischoff-Ferrari, Giovannucci, Willett, Dietrich, & Dawson-Hughes, 2006). Nevertheless, the available global data indicate an average serum 25(OH)D concentration of 50 nmol/L, suggesting that at least 50% of the population in those countries have an insufficiency of the vitamin (Lips, 2010). There appears to be a close link between chronic disease prevalence and intakes of nutrients like calcium and vitamin D (Holick & Chen, 2008; Peterlik, Boonen, Cross, & Lamberg-Allardt, 2009). However after reviewing the evidence available then, an expert consultation concluded that there was no evidence to favor an extraskeletal role for calcium or vitamin D (Institute of Medicine, 2011).

We have approached this chapter through a conceptual model that places obesity at the center of systemic inflammation, endothelial dysfunction, T2DM, and CVD (Fig. 1). Hence, obesity-related systemic inflammation drives endothelial dysfunction, which in turn is a risk factor for both T2DM and CVD (not shown). However obesity can impinge directly on all these conditions. The model is not strictly etiopathological in description but instead serves to conceptualize the flow of this review. With this in mind we present the potential role of calcium and vitamin D in obesity, inflammation, endothelial dysfunction, dyslipidemia, and T2DM. We cover potential mechanisms that contribute to these effects and provide an update on clinical trials that have investigated the area in the last five years.

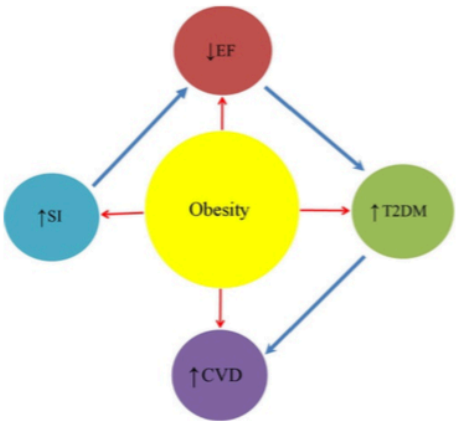

Figure 1 A framework of obesity and related chronic conditions that underpins the review. *SI*, systemic inflammation; *EF*, endothelial function; *T2DM*, type 2 diabetes mellitus; *CVD*, cardiovascular disease. ↓, decreased; ↑, increased.

2. ENERGY BALANCE AND OBESITY

A person is said to be in energy balance (EB) when energy intake (EI) equals energy expenditure, and body weight is stable over a defined period. More appropriately, EB is the difference between the rate of change in EI and the rate of change in energy expenditure. Obesity is hence a chronic positive EB and its presence is commonly ascertained through a variety of surrogate indices of fat stores like body weight and body mass index (BMI) to more sophisticated measures of total and regional distributions of adipose tissue (AT) mass such as DEXA. It has been proposed that even a positive balance of +50 kcals/d can result in significant amounts of body fat stores over time (Hill, Wyatt, Reed, & Peters, 2003). Such small aberrations also emphasize that, in general, the human body is capable of precise matching of intake to expenditure despite wide swings in both on a day-to-day basis (Martinez, 2000).

There are three components to total energy expenditure (TEE) and these are resting metabolic rate (RMR), diet-induced thermogenesis (DIT), and physical activity. In a sedentary person, they approximately contribute 60%, 10%, and 30%, respectively, to TEE. EI is the total kilojoules intake from a variety of food groups that contribute varying proportions of carbohydrate, protein, and fat. Alcohol consumption can also make a variable contribution in some individuals. When in EB and with stable body stores, the rates of oxidation of carbohydrate, protein, and fat in the body must match the rate of ingestion of these macronutrients. On a day-to-day basis, the individual balances of carbohydrate and protein are easily met since humans have the capacity to match the use of those fuels to the amounts ingested (Flatt, 1995). However this balance is not easily achieved with dietary fat, particularly saturated fat. High intakes of fat do not necessarily engender a higher fat oxidation rate (FOX) and so, in the short-term, result in a positive fat balance (Flatt, 1995). Overall, the ability to attain long-term EB is controlled by fat balance, and the only avenues available to influence the latter outcome is by controlling fat intake or increasing fat oxidation. It hence comes as no surprise that expert national and international committees for the control of obesity, emphasize a reduction in fat intake and an increase in physical activity so as to drive fat oxidation.

2.1 Calcium and Obesity

The first known link between calcium and body fatness came from a study by McCarron, Morris, Henry, and Stanton (1984) who showed an inverse

association between calcium intake and BMI. However much of the current impetus to this area was provided by Zemel and colleagues (Zemel, 1998; Zemel, Shi, Greer, Dirienzo, & Zemel, 2000; Zemel, Thompson, Milstead, Morris, & Campbell, 2004). Their proposition stemmed from a series of cellular and animal studies, and predicted that the concentration of intracellular calcium (iCa^{2+}) was central to the partitioning of fat through either lipogenic or lipolytic pathways. Accordingly, a low calcium intake would trigger higher parathyroid hormone (PTH) and/or 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which in turn would increase iCa^{2+}. A higher iCa^{2+} then stimulated lipogenesis but reciprocally inhibited lipolysis. A decrease in core body temperature possibly via reduced uncoupling protein 1 activity would also promote weight gain. Over the ensuing years, such a paradigm of events has been expanded to include other potential pathways. We present a conceptual scheme for the action of calcium and vitamin D, which also serves to underpin the sections covered in this chapter (Fig. 2). In essence, increases in calcium, vitamin D, or their combination may converge on interlinked pathways that regulate both EI and TEE (Fig. 2). Theoretically, suppression of PTH should also allow for similar effects, though to the best of our knowledge, nutritional studies have not tested such an approach. In this chapter we highlight the evidence to date in support or against a role for calcium, both from a mechanistic viewpoint and from an outcome-based approach.

2.2 Calcium, Thermogenesis, and Fat Oxidation

Thermogenesis or simply heat production is usually encountered in two settings: that due to meal ingestion and in response to a cold environment. Logically, they are termed DIT and cold-induced thermogenesis. DIT is the obligatory rise in EE on meal ingestion and varies with meal size, composition, and individual characteristics. An adaptive component that is driven by the sympathetic nervous system is also evident. A higher DIT for a given meal size then represents the propensity to dampen weight gain all other things being the same. The rise in insulin concentration on mixed meal ingestion serves to channel nutrient disposal toward storage rather than oxidation. So carbohydrate oxidation will rise but fat and protein oxidation rates will be suppressed. The net effect is whole body respiratory quotient (RQ, the ratio of CO_2 production/O_2 consumption) will rise immediately after a meal and then fall back to baseline. Overall, when comparing two or more meals, a lower RQ (as measured by the integrated area under the postprandial curve) signals a greater FOX relative to the control condition.

Indirect calorimetry together with measures of nitrogen excretion, also permits direct calculations of the rates of carbohydrate, protein, and FOX in both the fasted and fed states (Ferrannini, 1988).

We have investigated the influence of calcium and DIT in two acute randomized controlled trials (RCTs) (Chan She-Ping-Delfos & Soares, 2011; Cummings, James, & Soares, 2006). We have observed that FOX was higher following calcium whether the additional calcium came from a dairy source or whether it came from a pharmaceutical preparation. This was accompanied by lesser suppression of nonesterified fatty acids and to some extent glycerol, following both calcium sources. Overall higher calcium increased FOX and possibly increased triglyceride (TG) lipolysis (Cummings et al., 2006). In a complementary study, breakfast calcium was manipulated but lunch calcium was matched in a within-subject design (Chan She-Ping-Delfos & Soares, 2011). We observed higher overall DIT and FOX following calcium with significant effects on food intake. A review of the evidence led us to conclude that the effect of calcium on increasing FOX was very consistent (Soares, Murhadi, Kurpad, Chan She Ping-Delfos, & Piers, 2012). A meta-analysis by an independent group confirmed these findings through a metaregression that predicted a ~11% increase in FOX for every 800 mg/d increase in calcium intake across the trials in that analysis (Gonzalez, Rumbold, & Stevenson, 2012). To date there have been no long-term studies that have exploited this relationship. A recent short-term, crossover trial of two weeks did not find any beneficial effect of calcium on substrate oxidation (Gonzalez, Green, Campbell, Rumbold, & Stevenson, 2014). In part this may have arisen from the choice of physically active participants in whom the capacity to increase FOX in response to calcium would be less as they start from a higher baseline FOX, compared to their more sedentary counterparts.

2.3 Calcium and Fecal Fat

The absorption of dietary calcium is complex and occurs through both active uptake and passive diffusion around intestinal cells. Several factors govern the process and include an adequate vitamin D status, the amount of calcium ingested, its source and salt, as well as the interference of other dietary constituents. All factors considered, at a low calcium load the absorptive efficiency is high but this decreases exponentially to very low efficiency at very high loads (Heaney, Weaver, & Fitzsimmons, 1990). Hence at every occasion of calcium ingestion, there is a considerable amount left over in the

gastrointestinal (GI) tract lumen. This residual calcium is not without metabolic effect. In fact luminal calcium interacts with dietary fatty acids (FAs) to form insoluble calcium-FAs soaps, which are then excreted. After a meal, saturated FAs resided in the GI tract for a longer time than other types of FAs, and this would make them more available for the subsequent interaction with calcium. Calcium could also increase bile acid excretion and overall decrease the digestible energy from fat in the diet (Soares et al., 2012). Hence fecal excretion presents a pathway of impact on EB (Fig. 2), since it decreases fat absorption. Available data have now been synthesized to provide an estimate of an increased fecal fat excretion of 5 g/d for 1200 mg/d calcium consumed (Christensen et al., 2012). It is unclear whether such effects would result in measurable weight loss. However, they are expected to account in part for the observation of a lower TG and circulating lipid concentration following higher calcium intake, and so assist in the reduction of CVD risk (Lorenzen & Astrup, 2011; Major, Alarie, Dore, Phouttama, & Tremblay, 2007). Some recent confirmation comes from a cross over design of three dietary interventions, where an increased fecal fat following the dairy and cheese diets (each providing 1700 mg/d of calcium) was observed with a concomitant attenuation of total and LDL-cholesterol (Soerensen, Thorning, Astrup, Kristensen, & Lorenzen, 2014).

2.4 Calcium and Food Intake

A "calcium appetite" has been hypothesized as an innate driver for calcium-rich food sources when faced with calcium depletion (Tordoff, 2001). This highlights the potential importance of links between calcium intake and free-living food intake. The potential role of calcium intake on subjective cues of hunger and satiety as well as 24 h food intake have not been studied to a large extent. To date we found only eight studies reporting on these outcomes and they are summarized in Table 1. The studies were both acute—lasting a couple of hours—and chronic, lasting a year. Only two studies showed some effect that higher calcium intake may result in lowered EI. The acute study of Chan She-Ping-Delfos and Soares (2011) manipulated calcium intake at breakfast and followed it up with a standard lunch meal. Ad libitum intake at a buffet showed a trend to be lower and this pattern extended over the recalled dinner meal such that 24 h later the higher calcium arm had a significantly lower intake mainly due to decreases in carbohydrate and fat intake (Chan She-Ping-Delfos & Soares, 2011). Major et al. (2007) conducted a subgroup analysis of participants with low habitual

Table 1 Recent Trials Investigating the Effect of Calcium on Sensations of Hunger/Satiety and Prospective Food Intake

		Differences Between Treatments			
Authors and Year	**Study Design, Diets, and Duration**	**↓Hunger**	**↑Satiety**	**↓Intake at Buffet**	**↓24 h Intake**
Gunther et al. (2005)	PT ($n=26$). 2 diets of low (<800 mg/d) or high D (1000–1400 mg/d) for 1 yr	–	–	–	No
Jacobsen, Lorenzen, Toubro, Krog-Mikkelsen, and Astrup (2005)	CO ($n=10$). 3 dairy diets of 1800 mg/d (15% protein), 1800 mg/d (23% protein), and 500 mg/d (15% protein), each for 1 wk	No	–	No	No
Lorenzen et al. (2007)	CO ($n=18$). 4 meals 68 mg, 350 mg, 793 mg, and 850 mg of calcium over 7 h	No	No	–	–
Teegarden et al. (2008)	PT ($n=24$). 3 diets of 500 mg/d, 1400 mg/d (ND), or 1400 mg/d (D) each for 12 wk of weight loss	–	–	–	No
Major, Alarie, Doré, and Tremblay (2009)	PT ($n=63$). 600 mg calcium + 40 IU vitamin D or placebo over 15 wk of weight loss	–	–	Yes[a]	Yes, 1000 kJ
Chan She Ping-Delfos and Soares (2011)	CO ($n=11$). 2 breakfast meals 248 mg and 543 mg (D), followed by standard lunch 48 mg (D) over 8 h	No	No	Yes[b]	Yes, 2100 kJ/d
Gonzalez et al. (2014)	CO ($n=10$). 2 test meals of 3 mg calcium/kg or 15 mg calcium/kg weight, followed by standard exercise	Yes[c]	Yes[c]	–	–
Gonzalez, Ramos-Trautmann, Diaz-Luquis, Perez, and Palacios (2015)	CO ($n=20$). 4 test meals varying in calcium and protein intake	No	No	No[d]	–

CO, crossover; *d*, day; *D*, dairy calcium; *h*, hours; *ND*, nondairy calcium; *PT*, parallel trial; *wk*, week/s; *yr*, year; ↑, increase; ↓, decrease; –, not measured.
[a]In subset with low habitual calcium.
[b]Trend for lower intake.
[c]Only from composite appetite score.
[d]Increased intake following calcium.

calcium intake from a weight loss trial. They found that those on the high calcium arm had a significantly lower EI, due to lower fat intake. However a recent acute trial has suggested that acute calcium ingestion only transiently suppressed subjective appetite scores but resulted in an overcompensation of EI (Gonzalez et al., 2015). Potential mechanisms for these effects may arise through hormones like resistin (Kabrnová-Hlavatá et al., 2008), ghrelin (Gilbert et al., 2011), and leptin (Astrup, Chaput, Gilbert, & Lorenzen, 2010). There is some indication that GI tract hormones (Fig. 2) like glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are suppressed after calcium intake (Gonzalez et al., 2014). However these do not appear to be consistent in their effect since in a follow-up study the same group of authors did not find any difference in GLP-1 or GIP (Gonzalez et al., 2015). Overall there are too few studies of varying designs to allow confidence in the association between calcium intake and food intake or satiety. A more concerted research effort is required in this area, since suppression of hunger and reductions in EI are a more powerful approach to weight loss relative to increasing energy expenditure.

2.5 Calcium and Weight Loss

The interest in the regulation of body weight by calcium has seen a large amount of activity since it was first proposed. There are numerous cross-sectional studies that support the thesis that low calcium intake is associated with higher body fatness. A meta-analysis has indicated that for every 800 mg/d increase in calcium intake, a decrease in BMI of 1.1 kg/m^2 may be predicted (Dougkas, Reynolds, Givens, Elwood, & Minihane, 2011). This is similar to the outcome of another meta-analysis where a small but statistically significant effect on body weight was noted (Onakpoya, Perry, Zhang, & Ernst, 2011) but contrasts with the conclusion that calcium supplementation had no effect on body weight (Trowman, Dumville, Hahn, & Torgerson, 2007). However none of these studies provided information on body fat and causal evidence in favor was required. We therefore reviewed the evidence from RCTs available then (Soares, Chan She Ping-Delfos, & Ghanbari, 2011) and found that of 15 trials that met our criteria only 2 showed a significant increase in fat loss (Major et al., 2009; Zemel et al., 2004). A further two studies had sizeable differences of 1.8 kg (Riedt et al., 2007) and 2.2 kg (Shapses, Heshka, & Heymsfield, 2004) in the direction of the hypothesis but lacked the statistical power (Soares, Chan She Ping-Delfos, et al., 2011). Since many of the older trials in the

literature predated the calcium-body weight hypothesis, they were invariably designed with bone metabolism in mind. In the last three years a few new trials that address these concerns have begun appearing in the literature and we provide an update of all findings to date (Table 2). Clearly there is an ongoing need for more RCTs but two newer ones suggest an effect on fat distribution rather than total body fat (Rosenblum et al., 2012; Zhu et al., 2014) and one study reported similar benefits without an imposed caloric restriction (Tabesh et al., 2015).

Table 2 The Effect of Supplementing Calcium and Vitamin D on Body Weight, Fat Loss, and Its Distribution

Authors and Year	Study Design and Duration	Intervention	Outcome
Effect of calcium ± vitamin D and caloric restriction			
Trowman et al. (2007)	SR of RCTs for weight loss	Calcium ± vitamin D (various doses) vs placebo	No effect on weight loss
Soares, Chan She Ping-Delfos, et al. (2011)	Review of RCTs for weight loss from 2000 to 2011	Calcium ± vitamin D (various doses) vs placebo	No effect on fat loss
Onakpoya et al. (2011)	SR and MA of RCTS for weight loss	Calcium (various doses) vs placebo	Small but significant increase in weight loss and fat loss but not BMI
Smilowitz et al. (2011)	PT of 3 arms over 12 wk of caloric restriction	900 mg/d calcium vs 3–4 dairy serves/d vs placebo	No effect on weight, WC, or FM
Song and Sergeev (2012)	Review of RCTs	Calcium ± vitamin D (various doses) vs placebo	No effect
Thomas, Wideman, and Lovelady (2010)	PT of 2 arms over 16 wk of caloric restriction	<500 mg/d calcium plus exercise vs >1200 mg/d calcium plus exercise	No effect

Continued

Table 2 The Effect of Supplementing Calcium and Vitamin D on Body Weight, Fat Loss, and Its Distribution—cont'd

Authors and Year	Study Design and Duration	Intervention	Outcome
Rosenblum, Castro, Moore, and Kaplan (2012)	2 PT trials, each of 2 arms, over 16 wk of caloric restriction	1050 mg/d calcium plus 300 IU/d vitamin D vs placebo	No effect on weight loss; significant reduction in VAT in both trials independently and combined
Zhu et al. (2013)	PT of 2 arms over 12 wk of caloric restriction	600 mg/d elemental calcium plus 125 IU/d vitamin D vs placebo	Significant increase in fat loss and VAT loss
Tabesh, Azadbakht, Faghihimani, Tabesh, and Esmaillzadeh (2015)	RCT of 4 arms over 8 wk of free living	1. 50,000 IU/wk vitamin D3 plus 1000 mg/d calcium vs 2. 50,000 IU/wk vitamin D3 plus calcium placebo vs 3. 1000 mg/d calcium plus vitamin D3 placebo vs or 4. Vitamin D3 placebo plus calcium placebo	Significantly, lower BMI, hip, and systolic blood pressure with marginal waist effect ($p=0.08$) in calcium plus vitamin D groups

BMI, body mass index; *d*, day; *FM*, fat mass; *MA*, meta-analysis; *mg*, milligrams; *PT*, parallel trial; *RCT*, randomized controlled trials; *SR*, systematic review; *VAT*, visceral adipose tissue; *WC*, waist circumference; *wk*, weeks; *%FM*, percentage fat mass.

3. VITAMIN D

3.1 Physiology of Vitamin D

Originally termed D, vitamin D was the fourth known vitamin (Pilz et al., 2013). The main source of vitamin D is through endogenous production, whereby solar UV-B irradiates 7-dehydrocholesterol present in the skin (Liu, 2012; Pilz et al., 2013). The second source of vitamin D is dietary intake, and in general milk and dairy product intake make the greatest contribution. However the popular literature is filled with anecdotal evidence of the wonders of vitamin D and the multitude of over the counter preparations makes supplement usage a major source for vitamin D.

Once in the blood stream, vitamin D is transported by the vitamin D-binding protein (Ralph, Lucas, & Norval, 2013). Two hydroxylation

steps convert the inactive vitamin D to its active metabolite, $1,25(OH)_2D$ (Ralph et al., 2013). The first step occurs in the liver whereby vitamin D undergoes 25-hydroxylation to form 25(OH)D (Ralph et al., 2013). Then, the enzyme 1-alpha hydroxylase catalyzes the conversion of 25(OH)D to $1,25(OH)_2D$ (calcitriol) in the mitochondria of the kidney (Ralph et al., 2013). It is recognized that this enzyme is present not only in the kidneys, but also in the pancreas (Pilz et al., 2013) and immune cells including T cells, B cells, and macrophages (Bouillon et al., 2014; Hossein-Nezhad et al., 2013; Neve, Corrado, & Cantatore, 2013). Thus several cell types have the ability to locally produce $1,25(OH)_2D$ from 25(OH)D. The active metabolite can then bind to the nuclear vitamin D receptor (nVDR), where it forms a heterodimer with the retinoid X receptor (Pilz et al., 2013). This complex binds to certain DNA regions, called vitamin D responsive elements. Recruitment of coactivators and enzymes with histone acetylation activity results in structural changes in chromatin and facilitates gene transcription (Zhang et al., 2012). In fact, the VDR regulates approximately 3% of the human genome (~700 genes) (Pertea & Salzberg, 2010; Pilz et al., 2013). Nuclear VDRs are present in the majority of cells of the body and forms the basis for the investigations into extraskeletal benefits of vitamin D.

Maintaining adequate vitamin D levels is important for preventing rickets, osteoporosis, and fractures (Tran et al., 2013). Many also argue that a causal link between vitamin D insufficiency and other chronic diseases exists. However, the Institute of Medicine (2011) concluded that there was no support for a causal link between low vitamin D and nonbone endpoints. This conclusion was due to limited randomized placebo controlled clinical trials. Furthermore, few RCTs used an amount of vitamin D that raises the blood level above 75 nmol/L (Institute of Medicine, 2011). It has been opined that RCTs are "desperately needed" to evaluate the effects of vitamin D doses in the range of 2000–5000 IU/d on noncalcemic health outcomes (Holick et al., 2011).

3.2 Vitamin D and Obesity

An inverse relationship between high adiposity and low serum 25(OH)D is well established, regardless of whether the index used is BMI (Gonzalez et al., 2015; Young et al., 2009), total body fat mass (Caron-Jobin et al., 2011), waist circumference (Cheng et al., 2010; Gonzalez et al., 2015), subcutaneous AT (Caron-Jobin et al., 2011; Cheng et al., 2010; Young et al.,

2009), or visceral AT (Caron-Jobin et al., 2011; Cheng et al., 2010; Song & Sergeev, 2012; Young et al., 2009). As a result, there has been a tremendous interest in the potential for vitamin D to influence weight loss in obesity.

3.2.1 Sequestration and Volumetric Dilution of Vitamin D in Obesity

A consistent inverse relationship between low vitamin D status and indices of fatness, does not confirm the direction nor causality of the association (Ibero-Baraibar, Navas-Carretero, Abete, Martinez, & Zulet, 2014; Ortega et al., 2008). Vimaleswaran et al. (2014) employed a bidirectional Mendelian randomization analysis and concluded that while a high BMI was always associated with a low 25(OH)D, the reverse was less likely to be true. Possible reasons for such a conclusion may stem from a sequestration of the vitamin in adipose and other storage tissues, a volumetric dilution due to the solubility of the vitamin within AT or a combination of the two. The latter may also explain why obese individuals show a lesser increment in circulating 25(OH)D in response to oral vitamin D3 relative to their lean counterparts (Gallagher, Yalamanchili, & Smith, 2013).

Rosenstreich, Rich, and Volwiler (1971) proposed that AT was the major storage site of vitamin D, and that it was slowly released back into circulation. This storage allowed a stable level of vitamin D to be maintained and the slow release prevented flooding of a potentially toxic vitamin back into the circulation. Heaney, Horst, Cullen, and Armas (2009) confirmed that distribution of 25(OH)D was highest in fat mass (FM) (34%), followed by serum (30%) and then muscle (20%). However the mechanisms that facilitate the uptake and subsequent release of 25(OH)D from AT are still unknown (Malmberg et al., 2014). Sequestration hence implies that vitamin D is "bound" in AT and is not readily released into plasma (Worstman, Matsuoka, Chen, Lu, & Holick, 2000).

Volumetric dilution of vitamin D indicates that circulating concentrations vary as a function of body size, particularly AT, as the vitamin is fat-soluble. Drincic, Armas, Van Diest, and Heaney (2012) favored this explanation for their observations that body weight was the best single determinant of 25(OH)D and the relationship was best described by a hyperbola. They argued that adjustment for weight nullified the intersubject variability due to obesity, so a volumetric dilution explained why obese had lower levels of 25(OH)D relative to their lean counterparts. If this is true then with a reduction in body weight and hence AT, the circulating 25(OH)D levels should increase proportionately. We have recently conducted a systematic

review and metaregression of studies over the last 21 years. Our analysis indicated a marginally significant effect between body weight and %fat and 25(OH)D. For every 10% decrease in %fat there was a 10.5 nmol/L increase in 25(OH)D (Pannu, Zhao, & Soares, 2015). While this would support the volumetric dilution theory, the amount returning to the circulation is small, relative to the large storage capacity of AT, ie, ~103 nmol/kg of AT. There are many potential reasons for the lesser return of 25(OH)D during weight loss. These may include the requirement of 25(OH)D toward autocrine/paracrine function of AT (Calton, Keane, & Soares, 2015; Ding, Gao, Wilding, Trayhurn, & Bing, 2012), increased conversion of 25(OH)D to its inactive metabolites (Jones, Strugnell, & DeLuca, 1998; Wamberg, Christiansen, et al., 2013), and perhaps the protection of an individual from potential toxicity that may arise from a dramatic increase in status (Rosenstreich et al., 1971). Overall, we believe that the lower levels of 25(OH)D in obese are governed by both a sequestration and a volumetric dilution effect.

3.2.2 Vitamin D and Weight Loss

There are many potential pathways for vitamin D to impact on EB. These may include a stimulation of components of TEE, an increase in fat oxidation as well as a reduction in food intake (Fig. 2). In a cross-sectional study we have observed that 25(OH)D was a significant predictor of RMR after accounting for all traditional factors that contribute to between-subject variability in RMR (Calton, Pathak, et al., 2015). This amounted to a ~57 kJ/d higher RMR for every 10 nmol/L increase in 25(OH)D. So, theoretically improving status from 40 to 75 nmol/L would result in a ~200 kJ/d (50 kcals/d) increase in RMR, a clinically relevant amount for EB (Hill et al., 2003). Although one previous study implies a role in DIT, the added effect of calcium cannot be ruled out (Chan She-Ping-Delfos & Soares, 2011). Moreover we are unaware of studies reporting an effect of vitamin D on spontaneous physical activity in humans, though a relationship between outdoor activity and higher vitamin D status would be logically ascribed to increased sun exposure. More interesting is the potential of vitamin D to influence food intake (Fig. 2). Vitamin D may improve insulin sensitivity and circulating insulin is more powerful than glucose in modulating subjective hunger/satiety cues and ultimately food intake (Flint et al., 2007). However to the best of our knowledge there are no clinical

trials that have tested the effect of vitamin D per se on food intake and its regulation, which makes it a fertile area of investigation in the future.

There is now some exciting animal and cellular data that strongly implicate vitamin D and its receptors in EB. However their overall interpretation and extrapolation to human obesity is not straight forward (Pathak, Soares, Calton, Zhao, & Hallett, 2014). A role for the nVDR in energy metabolism was confirmed, when nVDR null mice gained less body fat on a high-fat diet through greater energy expenditure and fat oxidation (Narvaez, Matthews, Broun, Chan, & Welsh, 2009; Wong et al., 2009). It must be remembered that these were global knockout mice. When mice with an overexpression of VDR in AT were studied, the opposite outcome was observed, namely, greater obesity secondary to a decrease in both energy expenditure and FA oxidation (Wong et al., 2011). Furthermore, mice lacking the enzyme CYP27B1, which produces active vitamin D in the kidneys, also show lower body weight and less abdominal fat mass (Bouillon et al., 2014). Cellular models support a mechanistic role for $1,25(OH)_2D$ in adipocyte biology through both genomic and nongenomic actions. $1,25(OH)_2D$ can increase apoptosis, decrease adipogenesis (Sergeev, 2009; Song & Sergeev, 2012), and reciprocally control both lipolysis and lipogenesis (Shi, Norman, Okamura, Sen, & Zemel, 2001). So how do all these effects impinge on clinical weight loss?

There are two lines of questioning that emerge with regard to vitamin D and weight loss. Does supplementation of vitamin D accelerate weight and fat loss during caloric restriction? The evidence so far is rather meager since it includes one review and one recent RCT conducted for the purpose (Table 3). Overall, there is no consistent evidence in favor of vitamin D per se on weight loss or body composition. Perhaps, vitamin D works best with calcium (Table 2) or the circulating levels of 25(OH)D attained are not sufficient to impact on body weight. The second question addressed in this area, is whether increases in vitamin D status result in a decrease in obesity in the absence of caloric restriction? There were many more studies on the effect of vitamin D per se on body weight in the absence of any imposed caloric restriction. We recently reviewed the evidence in the literature on vitamin D supplementation in the absence of caloric restriction and found that vitamin D supplementation had no effect on body weight, FM, or body fatness (Pathak et al., 2014). However a trend for an effect ($p=0.09$) suggested that vitamin D may reduce BMI. Clearly, this needs confirmation but the available data suggest that increasing vitamin D per se does not result in a spontaneous change in body weight or composition as well (Table 3).

Table 3 The Effect of Supplementing Vitamin D on Indices of Body Fatness and Its Distribution

Authors and Year	Study Design and Duration	Intervention	Outcome
Effect of vitamin D and caloric restriction			
Soares, Chan She Ping-Delfos, et al. (2011)	Review of RCTs for weight loss from 2000 to 2011. 7 trials met criteria	Vitamin D (various doses)	
Mason et al. (2014)	PT of 2 arms over 1 yr of caloric restriction	2000 IU/d or placebo	No difference in weight loss. Subjects who achieved >80 nmol/L showed greater weight, WC, and FM loss
Effect of vitamin D without caloric restriction			
Mora et al. (2013)	SR and MA	Vitamin D alone (various doses)	MA showed no effect on BMI
Pathak et al. (2014)	SR and MA	Vitamin D alone (various doses)	MA showed marginal effect on BMI (<0.09) but no effect on FM, %FM, FFM
Sadiya et al. (2015)	PT of 2 arms over 24 wk and maintenance of further 24 wk	6000 IU/d vitamin D in phase 1 (12 wk), 3000 IU/d in phase 2 (12 wk), and 2200 IU/d in phase 3 (24 wk)	No effect on weight, BMI, WC, FM
Chandler et al. (2014)	RCT of 4 arms over 12 wk	1000 IU/d, 2000 IU/d, and 4000 IU/d vs placebo	No effect on BMI
Tabesh et al. (2015)	RCT of 4 arms over 8 wk	**1.** 50,000 IU/wk vitamin D3 plus 1000 mg/d calcium vs **2.** 50,000 IU/wk vitamin D3 plus calcium placebo vs **3.** 1000 mg/d calcium plus vitamin D3 placebo vs **4.** Vitamin D3 placebo plus calcium placebo	Significantly, lower BMI, hip, and systolic blood pressure with marginal waist effect ($p=0.08$) in calcium plus vitamin D groups

BMI, body mass index; *d*, day; *FFM*, fat free mass; *FM*, fat mass; *MA*, meta-analysis; *PT*, parallel trial; *RCT*, randomized controlled trials; *SR*, systematic review; *WC*, waist circumference; *wk*, weeks; *yr*, year; *%FM*, percentage fat mass.

One of the overarching issues in determining the extraskeletal roles for vitamin D has been a consensus on the optimal circulating level of 25(OH)D that needs to be achieved, and for how long that level needs to be maintained to see a benefit (Bischoff-Ferrari et al., 2006; Pathak et al., 2014; Soares, Chan She Ping-Delfos, et al., 2011). While this may well depend on the endpoint in question, some studies find that 75–80 nmol/L for at least three months may be required for improving insulin resistance (IR) (von Hurst, Stonehouse, & Coad, 2010) and reducing obesity (Mason et al., 2014). Such information needs to be incorporated into future trial design.

4. SYSTEMIC INFLAMMATION AND CHRONIC DISEASE

Excessive consumption of energy, sugar, saturated fat, and low intakes of healthy fats and antioxidants characterizes the westernized dietary pattern (Calton, James, Pannu, & Soares, 2014). This pattern of eating is now common not only in well-developed countries but also many Asian countries (Soon & Tee, 2014). Some of these nutrients serve as ligands for toll-like receptor 2 and 4 on the surface of macrophages and adipocytes, resulting in gene transcription of proinflammatory cytokines through activation of NFκB, p38 mitogen-activated protein kinase, and the c-jun N-terminal kinase signaling cascades. Constant stimulation of these pathways results in a chronic state of inflammation (Enos et al., 2013; Fig. 3). In contrast, beneficial nutrients induce antiinflammatory effects through several mechanisms including activation of cytosolic phospholipase A2 and COX-2 (Liu et al., 2014) and downregulation of NFκB pathway and decreased cytokine release (Buttari et al., 2014; Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014).

Obesity-related chronic health conditions such as CVD, T2DM, and some cancers are underscored by a low-grade chronic inflammation (Fig. 1). Atherosclerosis, the major cause of CVD, is a chronic inflammatory condition, whereby activated immune cells reside in the atherosclerotic lesion secreting proinflammatory cytokines (Frostegard et al., 1999). In T2DM, inflammatory changes occur in other cells and tissues of the body too such as the liver, pancreatic islets, the vasculature, and circulating leukocytes. Together, these changes suggest that inflammation participates in the pathogenesis of T2DM (Akash, Rehman, & Chen, 2013; Donath & Shoelson, 2011).

4.1 Vitamin D and Inflammation

Vitamin D is well recognized as a modulator of immune function. A plethora of studies demonstrate that vitamin D has an association with

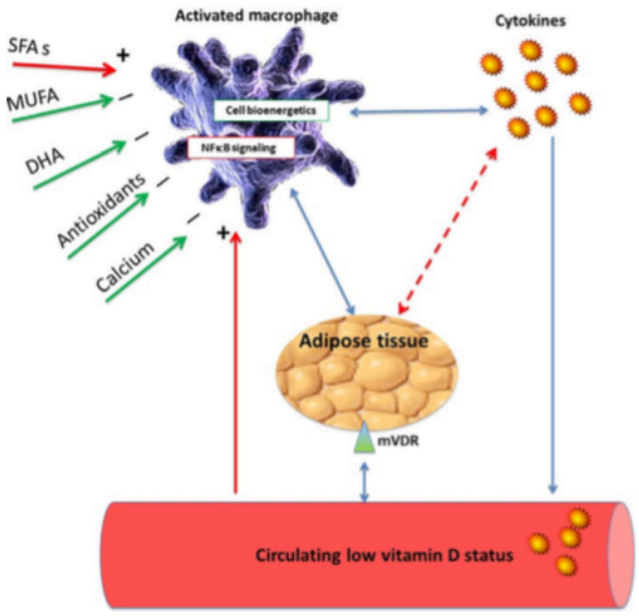

Figure 3 A conceptual scheme linking diet to inflammation. Release of proinflammatory cytokines is the net outcome of dietary components that stimulate (+) or inhibit (−) activation of circulating macrophages. This leads to a low-grade inflammation and immune cell infiltration into peripheral tissues, which is further perpetuated in individuals with low vitamin D status. Adipose tissue releases adipocytokines that further activate macrophages inducing a vicious proinflammatory cycle. *Adapted from Calton, E.K., Keane, K.N., & Soares, M.J. (2015). The potential regulatory role of vitamin D in the bioenergetics of inflammation.* Current Opinion in Clinical Nutrition and Metabolic Care, 18*(4), 367–373.*

autoimmune diseases (Adamczak, Nowak, Frydrychowicz, Kaczmarek, & Sikora, 2014; Goral, Brola, Kasprzyk, & Przybylski, 2015; Wang et al., 2015). Specifically, lower vitamin D levels have been found in rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, type 1 diabetes mellitus, multiple sclerosis, and autoimmune thyroid diseases (D'Aurizio, Villalta, Metus, Doretto, & Tozzoli, 2015). However, whether vitamin D has a role in the pathogenesis remains controversial, largely due to study design limitations such as seasonal variation of blood sampling, different vitamin D assay methods, inconsistent definitions of vitamin D deficiency, and population heterogeneity. Many studies also demonstrate an association between vitamin D and infections (Facchini, Venturini, Galli, Martino, & Chiappini, 2015) with the active metabolite promoting the innate immune response against infectious agents (Korf, Decallonne, & Mathieu, 2014). In

immune cells derived from humans, a strong antiinflammatory effect of the active metabolite is consistently observed. Table 4 shows that vitamin D has a clear antiinflammatory effect in 14 out of 16 studies in immune cells derived from humans. Interestingly, these findings cannot be replicated in data from RCTs (Table 5). There are several possible explanations that may clarify this discrepancy. First, cellular experiments manipulate only one or a few different cell types, so it may be that different cell types within the body respond differently to supplementation. If this is the case, any true beneficial effect may be diluted out in the whole body, resulting in non-detectable changes. Second, it is possible that participants must have a significant inflammatory condition that is much greater than the low-grade inflammatory state which characterizes obesity and related conditions.

There is limited human data examining the effect of calcium alone on inflammation. One secondary analysis found that in obese humans, dairy decreases plasma C-reactive protein (CRP) levels and raises antiinflammatory adiponectin. Mouse studies suggest that dietary calcium (from both supplements and dairy) has antiinflammatory effects (Zemel & Sun, 2008), whereby calcium inhibits oxidative and inflammatory stress in diet-induced obesity. Dietary calcium was shown to suppress adipocyte intracellular ROS production and NADPH oxidase gene expression and dairy inhibited ROS generation. Both calcium and dairy decreased inflammatory cytokine gene expression in AT and circulating plasma levels of TNF-α, IL-6, and MCP-1

Table 4 Evidence for an Antiinflammatory Benefit of Vitamin D on Human Immune Cells

Authors and Year	Cell Type and Vitamin D Type	Inflammatory Markers	Outcome
PBMCs			
Di Rosa et al. (2012)	Monocyte-derived macrophages and monocytes $1,25(OH)_2$	Monocytes: IL-1β, IL-6, TNF-α mRNA NC Macrophages + LPS: IL-1β, IL-6 mNRA NC TNF-α mRNA ↑ Macrophages + TNF-α: IL-1β mNRA NC IL-6 TNF-α mRNA ↓ Macrophages without stimulation: IL-1β, IL-6, TNF-α ↓	Monocytes: No effect Macrophages: Antiinflammatory

Table 4 Evidence for an Antiinflammatory Benefit of Vitamin D on Human Immune Cells—cont'd

Authors and Year	Cell Type and Vitamin D Type	Inflammatory Markers	Outcome
Zhang et al. (2012)	Monocytes $1,25(OH)_2$ 25(OH)D	IL-6 ↓ dose–response	Antiinflammatory
Ojaimi et al. (2013)	PBMCs Cholecalciferol	TNF-α, IL-6 ↓, then NC Unstimulated showed no effect as basal cytokine production was so low	Antiinflammatory (when serum levels >100 nmol/L)
Zhang, Leung, and Goleva (2013)	PBMCs-$CD14^+$ and $CD14^-$ T cells $1,25(OH)_2$	IL-6 ↓	Antiinflammatory
Cantorna, Snyder, Lin, and Yang (2015)	PBMCs $1,25(OH)_2$	INF-γ ↓ IL-4 ↑	Antiinflammatory
THP-1 cells			
Yang et al. (2012)	THP-1 $1,25(OH)_2$	IL-6, MCP-1 ↓	Antiinflammatory
Wang et al. (2014)	THP-1 $1,25(OH)_2$	MCP-1 ↓	Antiinflammatory
Tulk et al. (2015)	THP-1 $1,25(OH)_2$ 25(OH)D	IL-1β ↑	Proinflammatory (≥100 nmol/L 25(OH)D and ≥1 nmol/L $1,25(OH)_2D$)
Other			
Jain and Micinski (2013)	U937 monocytes $1,25(OH)_2$	IL-8, MCP-1 ↓	Antiinflammatory

$1,25(OH)_2$, 1,25-dihydroxyvitamin D; *25(OH)D*, 25-hydroxy vitamin D; *IL-4*, interleukin 4; *IL-6*, interleukin 6; *IL-8*, interleukin 8; *IL-1β*, interleukin 1β; *IFN-γ*, interferon gamma; *MCP-1*, monocyte chemotactic protein-1; *NC*, no change; *PBMCs*, peripheral blood mononuclear cells; *TNF-α*, tumor necrosis factor alpha; ↑, increase; ↓, decrease.

Table 5 Randomized Controlled Trials Examining the Impact of Cholecalciferol Supplementation on Inflammatory Markers

Authors and Year	Study Details (Dose, Duration, Health Status of Participants)	Outcome
Gepner et al. (2012)	Subject characteristics: $n=109$ Dose: 2500 IU/d Duration: 12 wk	hsCRP, NS
Sokol et al. (2012)	Subject characteristics: $n=90$, coronary artery disease Dose: 7142 IU/d Duration: 12 wk	hsCRP, IL-6, CXCL-10, IL-12, IFN-γ, NS
Wood et al. (2012)	Subject characteristics: $n=305$, healthy Dose: 0, 400 IU or 1000 IU/d Duration: 52 wk	hsCRP, IL-6, sICAM-1, NS
Yiu et al. (2013)	Subject characteristics: $n=100$, T2DM Dose: 5000 IU/d Duration: 12 wk	hsCRP NS
Wamberg, Kampmann, et al. (2013)	Subject characteristics: $n=43$, healthy Dose: 7000 IU/d Duration: 26 wk	hsCRP, IL-6, MCP-1, adiponectin, leptin, MMP-9, PAI-1, NS
Kampmann et al. (2014)	Subject characteristics: $n=15$, T2DM Dose: 11,200 IU/d 2 wk, 5600 IU 10 wk Duration: 12 wk	CRP, IL-6, IL-10 and TNF-α, NS
Sharifi, Amani, Hajiani, and Cheraghian (2014)	Subject characteristics: $n=53$, nonalcoholic fatty liver disease Dose: 3333 IU/d Duration: 16 wk	hsCRP trend $p=0.06$, TNF-α NS hsCRP in the placebo group ↑

d, day; *CXCL-10*, C-X-C motif chemokine 10; *hsCRP*, high-sensitivity C-reactive protein; *IFN-γ*, interferon gamma; *IL-6*, interleukin 6; *IL-10* interleukin 10; *IL-12*, interleukin 12; *NS*, nonsignificant; *MCP-1*, macrophage chemotactic protein 1; *MMP-9*, matrix metallopeptidase 9; *PAI-1*, plasminogen activator inhibitor-1; *sICAM-1*, soluble intracellular adhesion molecule 1; *T2DM*, type 2 diabetes mellitus; *TNF-α*, tumor necrosis factor alpha; *wk*, weeks; ↑, increase.

(Zemel & Sun, 2008). Clearly further RCTs are required to examine the impact of calcium alone on systemic inflammation.

5. ENDOTHELIAL DYSFUNCTION

The vascular endothelium is a monolayer at the interface between blood and tissue. It detects and reacts to blood-borne signals and changes in hemodynamic forces. In response to various stimuli including shear stress

at the endothelial cell surface, nitric oxide (NO) diffuses toward the lumen. Here, it acts to prevent both monocyte and platelet adhesion, and thereby attenuates inflammation and thrombosis. Hence endothelial dysfunction predisposes to both CVD and T2DM (Fig. 1). There are many ways to assess the functioning and integrity of the endothelium. Functional measures include assessing either coronary arteries or peripheral arteries for vascular reactivity. One noninvasive approach is measuring flow-mediated dilation (FMD) (Tousoulis, Antoniades, & Stefanadis, 2005). FMD occurs when endothelial cells release NO in response to a shear stress. Ultrasound is used to capture the change in artery diameter (Barac, Campia, & Panza, 2007) and this method is considered the gold standard for measuring endothelial dysfunction. Another noninvasive measure is arterial applanation tonometry. The method uses a sensitive probe applied to both the carotid and femoral arteries to study the characteristic of the wave form. A derived outcome is augmentation index (AIX) which is the ratio between the pulse pressures at the second systolic peak to that at the first systolic peak. Photo-plethysmographic recordings of the digital volume pulse are an easy to use bedside device, that outputs stiffness index (SI)—a measure of large artery stiffness, and reflective index (RI) that reflects small artery vascular tone (Woodman & Watts, 2003). Raised inflammatory markers such as CRP, cellular adhesion molecules (CAMs), vascular adhesion molecules, and E-selectin have also been used as markers of endothelial dysfunction (Alyami, Soares, Sherriff, & Mamo, 2014; Barac et al., 2007). We recently reviewed good quality RCTs and found that of ten such trials only two studies showed an improvement in endothelial function and a further three studies showed an effect on circulating markers (Alyami et al., 2014). We have updated the literature since then in Table 8. Of the studies retrieved, eight studies demonstrated no effect on endothelial function, with one study demonstrating a benefit in only one out of several markers of endothelial function. We found several RCTs of a very short duration (<6 weeks). Overall, the data are not consistently in favor of an effect on endothelial function. Future RCTs need to attain a higher absolute vitamin D status over a longer duration to determine whether vitamin D has a regulatory role in endothelial function.

6. VITAMIN D, DYSLIPIDEMIA, AND CARDIOVASCULAR DISEASE

CVD is predicted by raised total cholesterol, low levels of high-density lipoprotein cholesterol (HDL-C), high levels of low-density lipoprotein

levels, and elevated TG levels (Goodman, Hulley, Clark, et al., 1988; Hokanson & Austin, 1996; Parish et al., 2012). Atherosclerosis, the principal cause of CVD, is a process involving interplay among cells of the immune system and cells of the vessel wall. Endothelial dysfunction is considered an early marker of CVD and predicts CVD progression (Ross, 1993).

Vitamin D regulates a wide range of physiological and pathological processes which play a crucial role in atherosclerosis including vascular cell growth, cytokine expression, inflammatory pathways, and fibrotic pathways (Kassi, Adamopoulos, Basdra, & Papavassiliou, 2013). Importantly, 1,25(OH)D, the active vitamin D metabolite, can be produced locally by endothelial cells using serum 25(OH)D (Zehnder et al., 2002). Vitamin D has demonstrated antiatherogenic effects, and as a result, it has been speculated that vitamin D supplementation may be an inexpensive and safe intervention offering CVD protection (Breslavsky et al., 2013). This section will explore the evidence linking vitamin D status to traditional CVD risk factors like cholesterol and serum lipids.

A large body of observational studies have shown an association between low vitamin D status and adverse cardiovascular events (Dunnigan, Harland, & Fyfe, 1970; Forman et al., 2007; Ginde, Scragg, Schwartz, & Camargo, 2009; Kendrick, Targher, Smits, & Chonchol, 2009; Kim, Sabour, Sagar, Adams, & Whellan, 2008; Wang et al., 2008), even in the healthy general population (Kestenbaum et al., 2011; Wang et al., 2008). In contrast, high concentrations of 25(OH)D are associated with a favorable lipid profile (Jorde & Grimnes, 2011; Maki et al., 2009). However, older age, obesity, and ill-health are all contributory factors to low vitamin D levels, which may be responsible for the association observed. To date, the only prospective randomized trial that evaluated the effects of vitamin D supplementation on CVD events was the Women's Health Initiative (Hsia et al., 2007). However this study has been criticized for using an inadequate vitamin D dose of 400 IU/d and compliance was also low (Hsia et al., 2007). There is a need for well-designed prospective studies with adequate sample size, adequate vitamin D dose, high compliance, and appropriate length of follow-up.

A variety of mechanisms may explain this association between vitamin D and CVD, including a direct negative effect of vitamin D on IR and systemic low-level inflammation. Inflammatory cytokines have the ability to inhibit lipoprotein lipase, the enzyme responsible for hydrolyzing TG and very low-density lipoproteins (VLDLs) to form FAs and glycerol, thus leading to elevated TG and VLDL, and a decrease in HDL (Flores, 2005).

Vitamin D is able to regulate the production of proinflammatory cytokines at a cellular level (Table 6) and may counteract the adverse lipid profile caused by the proinflammatory markers. However, only evidence from RCTs can determine whether a causal relationship between low vitamin D levels and vascular disease exists. Six out of 12 studies demonstrated a beneficial effect on circulating lipids (Asemi, Saneei, Sabihi, Feizi, & Esmaillzadeh, 2015; Muñoz-Aguirre et al., 2014; Qin, Zhao, Chen, Yin da, & Wang, 2015; Salehpour et al., 2012; Schnatz et al., 2014; Tabesh et al., 2015), three studies showed no effect (Ponda, Dowd, Finkielstein, Holt, & Breslow, 2012; Witham, Dove, et al., 2013; Witham, Price, et al., 2013), two studies

Table 6 Impact of Vitamin D Supplementation on Endothelial Function

Authors and Year	Study Design and Duration	Outcome
Vitamin D		
Wood et al. (2012)	RCT for 1 yr	NS for sICAM-1
Gepner et al. (2012)	RCT for 16 wk	NS for FMD, PWV, or AIX
Stricker, Bianda, Guidicelli-Nicolosi, Limoni, and Colucci (2012)	RCT for 4 wk	NS for AIX
Sokol et al. (2012)	RCT for 12 wk	NS for E-selectin, hsCRP, RHI, RH-PAT
Arnson et al. (2013)	RCT for 5 d	VCAM-1 ↓ NS for sICAM-1, E-selectin, VEGF
Witham, Price, et al. (2013)	RCT for 1 yr	NS for arterial stiffness or FMD
Witham, Dove, et al. (2013)	RCT for 6 m	NS for RHI
Yiu et al. (2013)	RCT for 12 wk	NS for vascular function as determined by FMD or PWV
Ryu et al. (2014)	RCT for 24 wk Vitamin D + calcium vs calcium	NS for PWV or RAI

NS, nonsignificant; *AIX*, aortic augmentation index; *d*, day; *FMD*, brachial artery flow-mediated vasodilation; *hsCRP*, high-sensitivity C-reactive protein; *m*, month; *PWV*, pulse wave velocity; *RAI*, radial augmentation index; *RCT*, randomized controlled trial; *sICAM-1*, soluble intracellular adhesion molecule 1; *RHI*, reactive hyperemia index; *RH-PAT*, reactive hyperemia peripheral arterial tonometry; *VCAM*, vascular cell adhesion protein 1; *VEGF*, vascular endothelial growth factor; *wk*, weeks; *yr*, year.

showed a worsening of lipids (Pilz et al., 2015; Wang, Xia, Yang, & Peng, 2012), and one study showed mixed effects (Sollid et al., 2014; Table 6). While we acknowledge that more high quality RCT evidence is required, these outcomes at least indicate some benefit of vitamin D. However a very recent meta-analysis found that vitamin D increased cholesterol and TG, though they did acknowledge the considerable heterogeneity in the studies retrieved by their systematic review (Manousopoulou, Al-Daghri, Garbis, & Chrousos, 2015).

7. TYPE 2 DIABETES MELLITUS

According to the International Diabetes Federation (2013), 8.3% of adults or 382 million people worldwide have diabetes. This figure is projected to increase by 55% to over 592 million people by 2035. Of most concern are the 175 million individuals who are currently undiagnosed and progressing toward potential diabetes-related complications. Diabetes tends to be more prevalent in lower to middle income countries, with four out of every five people diagnosed with diabetes. In 2013, diabetes was estimated to cause 5.1 million deaths which equate to a mortality every 6 s (International Diabetes Federation, 2013). Certain ethnic groups, especially those from India and China, are at higher risk of T2DM than others (Chiu, Cohan, Lee, & Chuang, 2000; Gujral, Narayan, Kahn, & Kanaya, 2014; Misra et al., 2010; Shaw, Sicree, & Zimmet, 2010). There are a few key reasons for this increased risk including specific genes may increase the risk of T2DM through influencing beta cell dysfunction and IR (Ferrannini, 1998; Florez, 2008), and a higher tendency of central adiposity. Obesity is a primary driver of T2DM pandemic (Fig. 1), since approximately 60–90% of diabetes incidence is the result of higher body weight (Anderson, Kendall, & Jenkins, 2003).

7.1 Calcium Intake and T2DM

Prospective studies have found varying results between calcium intakes and risk of T2DM (Colditz et al., 1992; Liu et al., 2005; Pittas et al., 2006; van Dam, Hu, Rosenberg, Krishnan, & Palmer, 2006) (Table 7). A large prospective cohort study in 41,186 subjects found that higher calcium intake was not associated with risk of T2DM. In contrast, those who consumed calcium supplements had a decreased risk of T2DM compared to non-supplement users. However, within supplement users there was no association between the amounts or duration of calcium supplement and a lower

Table 7 The Effects of Supplementing Calcium and Vitamin D on Insulin Resistance and Type 2 Diabetes: Summary of Systematic Reviews and Meta-Analysis

Authors and Year	Study Design and Duration	Intervention	Outcome
Vitamin D and calcium			
Pittas, Lau, Hu, and Dawson-Hughes (2007)	SR and MA of observational studies and clinical trials from 1994 to 2007	Vitamin D ± calcium	No effect on T2DM
Pittas et al. (2010)	SR of various trials from 1984 to 2009	Vitamin D ± calcium	No effect on FPG, HbA1c, T2DM incident
Mitri, Muraru, and Pittas (2011)	SR and MA on observational studies and RCTs from 1984 to 2010	Vitamin D ± calcium	Vitamin D only MA: Significant decrease in risk of developing T2DM in >500 IU/d vs <200 IU/d (3 trials) Improved IR (2 trials) SR: RCTs no effect Vitamin D ± calcium—no effect
Vitamin D			
George, Pearson, and Witham (2012)	SR and MA of RCTs from 1984 to 2011	Vitamin D (various doses) vs placebo	Small but significant decrease FPG Small but significant improvement IR No effect on Hba1c
Seida et al. (2014)	SR and MA on RCTs from 1984 to 2013	Vitamin D	No effect on IR, Hba1c, FPG, T2DM incident, beta cell dysfunction
Nigil Haroon, Anton, John, and Mittal (2015)	SR on RCTs and longitudinal studies from 2003 to 2014	Vitamin D	Short-term studies (<3 months): supplementation may have positive impact on glycemic control, IR, beta cell dysfunction Long term (>3 months): no effect on Hba1c, beta cell function, IR

d, day; *FPG*, fasting plasma glucose; *Hba1c*, glycated hemoglobin; *IR*, insulin resistance; *MA*, meta-analysis; *RCT*, randomized controlled trial; *SR*, systematic review; *T2DM*, type 2 diabetes mellitus.

risk of T2DM (van Dam et al., 2006). Another large prospective study found opposing results with total calcium intake being inversely associated with incident T2DM. Those who consumed ≥1200 mg calcium via diet and supplements had a 21% reduced risk of development of incident T2DM than

those who consumed <600 mg/d. When looking at daily calcium intake via supplements only, there was an 18% lower risk of diabetes in those who consumed ≥500 mg vs those who consumed ≤250 mg (Pittas et al., 2006). A meta-analysis of these two prospective studies (Pittas et al., 2006; van Dam et al., 2006) found an 18% decrease in risk for incident T2DM in the highest (661–1200 mg) vs the lowest calcium (219–600 mg) intake groups (Pittas et al., 2007). Though evidence on calcium intake and risk of diabetes is conflicting, there does appear to be a potential link between the two. Optimal intakes of calcium in order to reduce risk of T2DM have not been confirmed, however a meta-analysis indicates calcium intakes of more than 600 mg/d are desirable, with intakes over 1200 mg/d being ideal (Pittas et al., 2007). Inconclusive evidence around calcium intake via supplements and risk of T2DM requires high quality RCTs to indicate a casual link.

7.2 Vitamin D and T2DM

Vitamin D has numerous endocrine and autocrine roles however one of the key roles of the vitamin is to maintain phosphate and calcium homeostasis via enhancing absorption in the gut (Cavalier, Delanaye, Souberbielle, & Radermecker, 2011). The importance of vitamin D is indicated by the presence of the VDR in numerous tissues, and the vitamin D system regulating around 3% of the human genome (Bouillon, Bischoff-Ferrari, & Willett, 2008). The expression of the VDR in pancreatic beta cells supports its physiological role of vitamin D in beta cell function (Bouillon et al., 2008; Jorde et al., 2012; Takiishi, Gysemans, Bouillon, & Mathieu, 2010).

7.2.1 Observational and Cross-Sectional Studies

A number of prospective (Forouhi, Luan, Cooper, Boucher, & Wareham, 2008; Husemoen et al., 2012), cross-sectional (Chonchol & Scragg, 2007; Del Gobbo, Song, Dannenbaum, Dewailly, & Egeland, 2011; Kayaniyil et al., 2010; Liu et al., 2009; Lu et al., 2009; Scragg, Sowers, & Bell, 2004) human studies (Chiu, Chu, Go, & Saad, 2004) have indicated an inverse association between 25(OH)D levels and risk of T2DM. A recent meta-analysis of 15 prospective studies indicated a significant inverse association between 25(OH)D levels and risk of T2DM, when comparing the highest 25(OH)D category vs the lowest category (Song et al., 2013). In addition, every 10 nmol/L increment in 25(OH)D reduced the risk of T2DM by 4%. There appeared to be a significantly lower risk of T2DM when 25(OH)D status reached 50 nmol/L. Institute of Medicine (2011) recommends a 25(OH)D status of 50–75 nmol/L for good bone health

however sufficient 25(OH)D levels for T2DM prevention have not been confirmed. One clinical study indicated a positive association between 25(OH)D levels and insulin sensitivity, and a negative association with plasma glucose concentration (Chiu et al., 2004). A negative effect of low 25(OH)D levels on beta cell function was indicated by the relationship between 25(OH)D and plasma glucose concentration. This suggests that those with lower 25(OH)D levels affected beta cell function by inhibiting the usual compensatory insulin response that would control glucose concentrations. Thus those with lower 25(OH)D level may have inhibited beta cell function thus higher plasma glucose levels. Similarly, a cross-sectional study found that those in the highest tertile of 25(OH)D (64 nmol/L) had significantly decreased fasting plasma glucose, fasting plasma insulin, and IR as compared to those in the lowest tertile of 25(OH)D (30 nmol/L) (Liu et al., 2009). Another study indicated that there was a fourfold increase in the risk of having T2DM in those in the lowest quartile ($\leq$43.9 nmol/L) of vitamin D compared to those in the highest quartile ($\geq$80 nmol/L) (Scragg et al., 2004). This indicates the critical role that vitamin D has by affecting insulin sensitivity, beta cell function, or both, thus low vitamin D levels are a potential risk factor for developing T2DM. However, the impact of other physiological mechanisms may also affect the risk of T2DM.

7.2.2 Randomized Controlled Trials

With vitamin D deficiency being a potential risk factor in developing T2DM, vitamin D supplementation would be a plausible solution. Unfortunately recent meta-analysis indicated that vitamin D supplementation trials are inconsistent in showing any changes in diabetes risk or glucose intolerance (Table 8) (George et al., 2012; Mitri et al., 2011). One meta-analysis indicated that there was no significant effect of supplementation on fasting plasma glucose, HbA1c, and IR. When looking at studies with subjects who already had impaired glucose tolerance or T2DM, a small but significant effect was found with supplementation and fasting plasma glucose, and IR. Thus the author states that the results are of debatable clinical significance (George et al., 2012). A second meta-analysis looked at those with glucose intolerance and those with T2DM separately. Results indicated no effect on glycemic outcomes in those with T2DM however there was an improvement in IR in those with glucose intolerance (Mitri et al., 2011). A third meta-analysis (Seida et al., 2014) also found no significant effect of vitamin D supplementation on glucose homeostasis or diabetes prevention. A number of limitations were identified in these reviews including a limited number of trials (George et al., 2012; Mitri et al.,

Table 8 The Effect of Supplementing Vitamin D on Cholesterol and Triglyceride Levels

Authors and Year	Study Design	Intervention	Outcome
Vitamin D			
Salehpour et al. (2012)	RCT, 12 wk	Vitamin D vs placebo	Improved HDL, LDL
Wang et al. (2012)	MA of RCTs	Vitamin D ± calcium vs placebo	Worsens LDL cholesterol concentrations, but does not appear to significantly affect total cholesterol, HDL, and TG
Ponda et al. (2012)	RCT, 8 wk	Vitamin D vs placebo	No effect on LDL, HDL, TC, or TG
Witham, Dove, et al. (2013)	RCT, 24 wk	Vitamin D vs placebo	No effect on cholesterol
Witham, Price, et al. (2013)	RCT, 52 wk	Vitamin D vs placebo	No effect on cholesterol
Muñoz-Aguirre et al. (2014)	RCT, 24 wk	Vitamin D vs placebo	Improved TG, no impact on TC, LDL, or HDL
Sollid et al. (2014)	RCT, 52 wk	Vitamin D vs placebo	Improved TC and LDL, HDL worsened
Qin et al. (2015)	RCT, 24 wk	Vitamin D vs placebo, + statins	Improved TC and TG
Pilz et al. (2015)	RCT, 26 wk	Vitamin D vs placebo	Worsened TG
Vitamin D and calcium			
Tabesh et al. (2015)	RCT, 8 wk	Calcium with vitamin D vs placebo	Improved LDL and HDL
Schnatz et al. (2014)	RCT, 104 wk	Vitamin D + calcium vs placebo	Improved LDL
Asemi, Foroozanfard, et al. (2015)	RCT, 8 wk	Calcium with vitamin D vs placebo	Improved TG, no impact on TC, LDL, or HDL

HDL, high-density lipoprotein cholesterol; *LDL*, low-density lipoprotein cholesterol; *MA*, meta-analysis; *RCT*, randomized controlled trial; *TC*, total cholesterol; *TG*, triglycerides; *wk*, weeks.

2011), small number of patients (George et al., 2012; Mitri et al., 2011; Seida et al., 2014), variable study quality (George et al., 2012; Nigil Haroon et al., 2015; Seida et al., 2014), suboptimal dosing of vitamin D (George et al., 2012; Seida et al., 2014), type of vitamin D supplementation (oral vs intramuscular, ergocalciferol vs calcitrol or cholecalciferol) (Nigil Haroon et al., 2015), and short treatment period and duration of follow-up (Nigil Haroon et al., 2015; Seida et al., 2014). In conclusion vitamin D deficiency may increase IR and reduce insulin secretion from beta cells. However the effect of vitamin D supplementation on reducing the risk of T2DM is inconclusive.

7.2.3 Parathyroid Hormone and T2DM

A key hormone in the vitamin D and calcium story is PTH secreted by the parathyroid glands. PTH is released in response to low serum calcium levels and mobilizes calcium from bone to maintain serum calcium. PTH also regulates the conversion of 25(OH)D to its active form, calcitrol, which in turn serves to increase calcium absorption. Despite this close interrelationship, it is unclear how PTH impacts on T2DM. Some studies indicate a decreased capacity to secrete PTH in T2DM resulting in lower circulating levels of the hormone (Ishida et al., 1993; Paula, Lanna, Shuhama, & Foss, 2001). However, with decreasing levels of vitamin D, the parathyroid glands are overly stimulated, resulting in secondary hyperparathyroidism (Holick et al., 2005; Holick, 2006). High PTH can impair glucose metabolism (Procopio & Borretta, 2003) and decrease insulin sensitivity (Chiu et al., 2000) which may explain its link with higher rates of T2DM (Gerich, 1998; Taylor & Khaleeli, 1997). However this contrasts with other data indicating no relationship between PTH and IR (Del Gobbo et al., 2011; Rueda, Fernandez-Fernandez, Romero, de Osaba, & Vidal, 2008). Clearly the interaction of vitamin D and PTH needs to be studied in concert to provide more insight into the area. While vitamin D may have a direct benefit on extraskeletal health, it is more than likely that such effects are seen only when PTH levels are normalized. Interestingly, one cross-sectional study found PTH was better than 25(OH)D in predicting insulin sensitivity and MetS both before and after weight loss (Soares, Ping-Delfos, et al., 2011).

8. SUMMARY AND CONCLUSION

In this chapter we have provided a framework to understand the role of calcium and vitamin D in obesity and chronic disease. There are

mechanistic pathways that favor an effect of dietary calcium on body weight regulation and these included whole body fat oxidation and fecal fat excretion. Mechanisms that show promise are calcium's effects on hunger/satiety and food intake and the potential thermogenic effect of vitamin D. Despite these beneficial effects, the balance of current evidence does not convincingly favor calcium or its combination with vitamin D for acceleration of fat loss. Normalization of calcium status possibly favors the attenuation of secular weight gain, which is important from a public health viewpoint but may not interest those looking for a magic pill.

Vitamin D status is consistently lower in overweight and obese and this is most likely a combined effect of a sequestration and a volumetric dilution phenomenon. For these reasons, supplementation of vitamin D needs to be quite high to achieve the desired plasma value. However determining a plasma level of 25(OH)D that would have beneficial effects across all chronic diseases is not an easy task. There certainly needs to be some international consensus on the target values to be achieved so as to direct future research. It is also unclear for how long this achieved status has to be maintained to see measurable effects. For these reasons, the RCT evidence is equivocal on a beneficial role for vitamin D in weight loss, inflammation, and endothelial dysfunction. However a small positive effect on insulin sensitivity and perhaps in T2DM can be deduced.

There is an urgent need for high quality trials to increase vitamin D status well above recommendations aimed at normality of bone function. We propose that normalization of calcium intake as per current national guidelines together with a 25(OH)D > 80 nmol/L maintained over at least six months is the way forward. When sufficient data becomes available, a clearer picture will emerge on the extraskeletal health benefits of these nutrients.

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Appendix B Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects: a systematic review and metaregression analysis

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Review

Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects: a systematic review and metaregression analysis☆,☆☆

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ABSTRACT

The purpose of this review was to confirm a volumetric dilution of vitamin D in obesity. It was based on the hypothesis that weight loss, particularly fat loss, would increase serum 25-hydroxyvitamin D (25OHD) in the obese. We conducted a systematic review of the literature over the last 21 years and included human trials that reported changes in 25OHD, weight, or body composition after weight loss. Study arms were excluded if vitamin D was supplemented, dietary intake exceeded 800 IU/d, or extreme sun exposure was reported. Eighteen of 23 trials that met our criteria documented an increase in vitamin D status with weight loss. Metaregression analyses indicated a marginally significant effect of weight loss on unadjusted weighted mean difference of 25OHD (β = −0.60 [95% confidence interval {CI}, −1.24 to +0.04] nmol/L; P = .06) and after adjustment for study quality (Jadad score ≥3) (β = −0.64 [95% CI, −1.28 to +0.01] nmol/L; P = .05). The effect of percent fat mass on weighted mean difference of 25OHD was also marginally significant before (β = −0.91 [95% CI, −1.96 to +0.15] nmol/L; P = .08) and after adjustment of study quality (β = −1.05 [95% CI, −2.18 to +0.08] nmol/L; P = .06). Collectively, these outcomes support a volumetric dilution of vitamin D. The slopes of the respective regression lines, however, indicate a smaller increase in 25OHD than would be expected from a direct mobilization of stores into the circulation. Hence, sequestration of 25OHD and its conversion to inactive metabolites would also play a role. Future studies could relate changes in body fat compartments to the enzymatic regulation of 25OHD in response to weight loss.

Abbreviations: %FM, percent fat mass; 1,25 dihydroxyvitamin D3, 1,25(OH)2D3; 25OHD, 25-hydroxyvitamin D; AT, adipose tissue; BIA, bioelectrical impedance analysis; BMI, body mass index; CBA, competitive protein binding assays using rachitic rat kidney cytosol; CI, confidence interval; CLIA, chemiluminescence immune assay; DEXA, dual-energy x-ray absorptiometry; ECLIA, electrochemiluminescent immunoassay; ELISA, enzyme-linked immunosorbent assay; FFM, fat free mass; FM, fat mass; LC-MS, liquid chromatography mass spectrometry; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; RCT, randomized controlled trial; RIA, radioimmunoassay; VDR, vitamin D receptor; WMD, weighted mean difference.

☆ Major finding: The data support a volumetric dilution of vitamin D in obesity but do not discount a sequestration effect.

☆☆ Model used: Systematic review of trials on human subjects.

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1. Introduction

Vitamin D and parathyroid hormone are essential for calcium homeostasis and bone metabolism [1]. There is accumulating evidence that vitamin D plays an important role in extraskeletal health and diseases such as diabetes mellitus, cancers, cardiovascular disease, and autoimmune disorders [2-5]. Serum 25-hydroxyvitamin D (25OHD) is the best clinical indicator of vitamin D status [1], and based on current cutoffs, the prevalence of vitamin D insufficiency worldwide is high. The escalating obesity crisis potentially contributes to this increasing incidence of vitamin D insufficiency because obese individuals have lower levels of 25OHD than their nonobese counterparts [6-8]. In fact, inverse associations among body weight, body mass index (BMI), and measures of body fatness with vitamin D status have been found across the lifespan [4,7,9,10]. Differences in 25OHD levels can be attributed to age, race, geography, skin color, habitual clothing, and sun exposure among other factors [11]. However, as vitamin D is fat soluble, it is commonly considered that the lower levels in the obese could also be due to uptake by adipose tissue (AT) and its clearance from plasma.

Rosenstreich et al [12] were the first to propose that AT was the major storage site of vitamin D and that its release from this tissue was quite slow. Based on the available evidence from animals and man, Heaney et al [13] have confirmed that the distribution of 25OHD was highest in fat mass (FM) (34%), followed by serum (30%) and then muscle (20%). Worstman et al [14] instead referred to "sequestration" of 25OHD for their observation that ultraviolet B radiation resulted in a significant increase in serum vitamin D3 in nonobese compared to obese individuals. This implied that vitamin D "disappeared" into AT and other tissues and was not immediately available in plasma for further metabolic activity. Such a phenomenon would account for the lower bioavailability of the vitamin in the obese [14]; however, the mechanisms controlling the deposition and release of vitamin D from AT are still unknown [15].

Drincic et al [16], however, support the theory of volumetric dilution, which implies that plasma levels of the vitamin decrease as body size and hence fat stores increase. It follows that, if fat stores decrease, there ought to be a greater return of vitamin D into plasma resulting in increased vitamin D status. In a cross-sectional study, Drincic et al [16] identified body weight as the single strongest predictor of 25OHD levels, followed by FM. Their best fitting model relating 25OHD and body weight was a hyperbola, which indicated that body weight explained 13% of the variance in 25OHD. A visual inspection of the regression line shows that the slope is steeper at a body weight less than 90 kg but gets progressively shallower at higher body weights [16]. Hence, an obese individual of 100 kg would need to lose a considerable amount of weight to benefit from an appreciable increase in 25OHD. The results of a clinical trial would support this interpretation because categories of weight loss less than 15% of baseline brought about increases of 5.3 to 8.3 nmol/L in 25OHD, whereas above a value of 15%, there was more than a doubling of this effect [17]. A caveat to such expectations would be the extensive conversion of released vitamin D to metabolites other than 25OHD, which would not be detected by the specific 25OHD assay used (Fig. 1).

It is unclear how 25OHD is handled once taken up by different body tissues such as AT and skeletal muscle. Both tissues are metabolically active, and the vitamin D receptor (VDR) is expressed in them [18,19]. Hence, a paracrine role in these tissues may account for some of the sequestration effect. Alternatively, if these tissues merely act as a store for the vitamin, then a sizeable amount would be available for release into plasma after tissue mobilization in response to weight loss [20]. There is also the possibility that both sequestration and volumetric dilution coexist in obese individuals. In Fig. 1, we schematically depict the basis of this review and the potential storage and release of 25OHD in an obese individual during weight loss. We focused on the larger stores of AT seen in overweight/obese individuals to allow for the best chance for hypothesized effects. We also negated the contribution from external sources of vitamin D by excluding study arms with vitamin D supplementation and those that reported excessive sunlight exposure during their trials. In this systematic review, we embarked on the hypothesis that weight loss without supplementary vitamin D would result in an increase in plasma 25OHD. We entertained the possibility that changes in 25OHD might be explained by volumetric dilution effect, sequestration effects, or other mechanisms (Fig. 1).

1.1. *Systematic review*

The aim of the search was to identify trials with weight loss that measured change in vitamin D status, but without vitamin D supplementation. Accordingly, placebo arms of trials that used vitamin D supplementation were included because we were only interested in relating the change in the 2 variables. Studies were identified through a systematic electronic search of Web of Science and PubMed Central databases over the period January 1994 to October 2015. One author (PP) conducted the search using the following terms: vitamin D, vitamin D-3, 25-hydroxy-vitamin D, 25-hydroxyvitamin D, serum 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D3, 25OHD, cholecalciferol, 25-OH vitamin D, 25-hydroxycholecalciferol, or serum 25OHD, and obese, overweight, caloric restriction, weight loss, fat mass, fat free mass, body mass index, BMI, or adipose tissue. Only articles published in the English language were included.

At the identification stage, the abstract was read, and the articles were selected, according to the following inclusion criteria: human clinical trials, weight loss study (through energy restriction, increased physical activity, or both), measurement of weight loss or body composition, study or placebo arm(s) without vitamin D supplementation, overweight/obese subjects, and change in serum 25OHD. Exclusion criteria included the use of the following terms in the abstract: vitamin D supplementation in all study arms, vitamin D–enriched foods greater than 800 IU/d, animal studies, gastric bypass/bariatric surgery studies, and duplicates of the same article retrieved from the 2 different databases. At the screening stage, the full text was read, and articles were screened based on the following inclusion criteria: change in serum 25OHD measured, included data

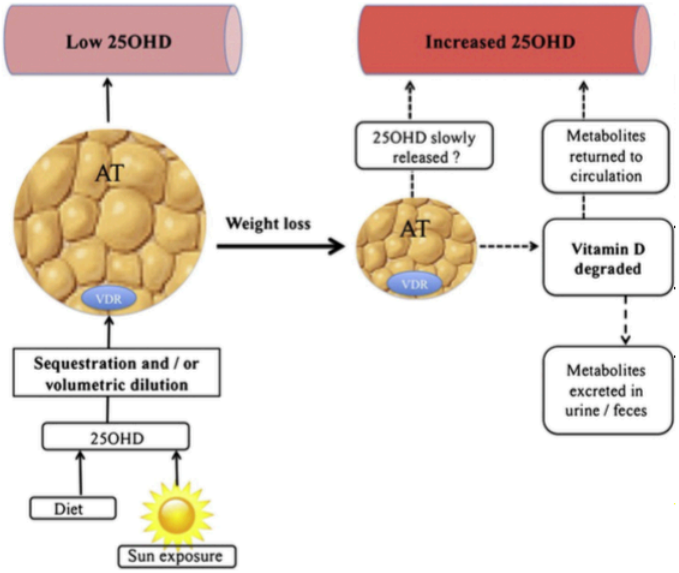

Fig. 1 – The potential pathways influencing 25OHD in obese individuals before and after weight loss. Solid lines in arrows indicate established mechanisms; dashed lines in arrows indicate potential mechanisms.

for at least 1 index of weight change, and weight loss as the primary or secondary outcome. Articles were excluded if vitamin D supplements were used, diets included foods enriched with vitamin D to result in greater than 800 IU/d, or extreme exposure to sunlight was indicated. Additional studies were sourced by manually searching the reference list and included 2 published studies from our laboratory [21,22]. After eligibility was determined, all randomized controlled trials (RCTs) were graded for their quality according to the Jadad score, with values greater than or equal to 3, indicating a high quality study [23], whereas 9 single-stranded studies were graded as zero. The overall process is outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocols (PRISMA) [24] flow diagram (Fig. 2).

Data extraction was carried out by 1 investigator (PKP) on an excel spreadsheet developed by the statistician (YZ). Another investigator (MJS) cross-checked quality criteria assessment and data entry. Any discrepancies were reviewed and discussed. The change in mean and SD was calculated for body weight, FM, fat free mass (FFM), or BMI, for studies that provided only prevalues and postvalues. Where necessary, vitamin D intake data were converted to international units per day; and 25OHD status, to nanomoles per liter. Fat mass was extracted as percentages; and FFM, as kilograms, as most articles presented their data in this manner. All subjects were overweight or obese at baseline; thus, FM (percentages) is an appropriate measure during weight loss studies. Furthermore, it is common to use FM (percentages) and FFM (kilograms) to evaluate nutrition status [25].

2. Methods and materials

2.1. *Statistical analysis*

2.1.1. *Meta-analysis main effects*

The primary outcome was the relationship of change in vitamin D status and change in weight/obesity status. The change in vitamin D status was calculated as postvalue minus the prevalue where a positive value implied an increase in the 25OHD status. Changes in the 4 main factors of interest in our article, (i) weight (kilograms), (ii) BMI (kilograms per square meter), (iii) FM (percentages), and (iv) FFM (kilograms), were also calculated as postvalue minus the prevalue; hence, a negative value implied a reduction in the 4 main factors. Some RCTs had multiple treatment arms. Each arm was included as a separate study in the meta-analysis. Both fixed-effects and random-effects meta-analysis models were carried out to obtain the weighted mean difference (WMD) of vitamin D status based on the studies included, to extrapolate results to the general population. To test for heterogeneity and identify the potential sources of heterogeneity, I^2 statistics and Galbraith plot were used. Potential publication and small sample size bias were assessed by visual inspections of funnel plots and Egger test.

2.2. *Confounders*

Potential confounders considered for analyses were mean age of subjects, percentage of females in each trial, duration of

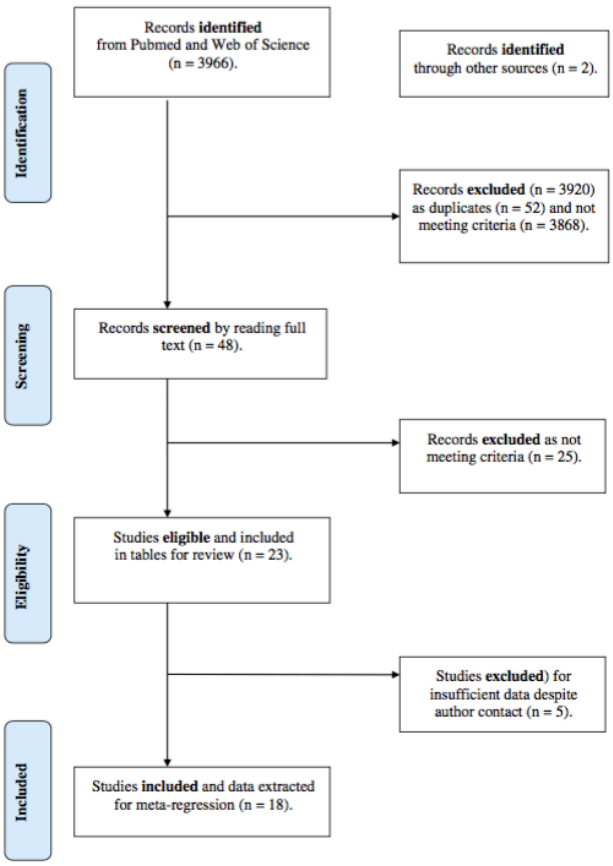

Fig. 2 – PRISMA flow diagram for vitamin D status and weight loss.

trial, vitamin D content in food (international units per day), total vitamin D intake in trial (international units per day × duration of trial), and season [26,27]. In our experience, age and sex (percentage of females) are potential confounders as they worked in opposite directions in regard to the effect of vitamin D supplementation on body weight [26]. In this article, variations in 25OHD between sexes may be due to differences in body composition, with women having a higher percentage of FM [6,28]. Duration of intervention may make a contribution to 25OHD because greater weight loss can be expected over a longer intervention period [29]. An effect of season is potentially intertwined with duration of study. Weight loss studies commencing in winter and lasting over summer may result in an increase in 25OHD due to season that will confound the expected increase due to greater weight loss [30]. However, we could not control for season as this was stated in only 4 studies [22,31-33]. The trials included in this review had also used a range of assays to measure 25OHD. These included radioimmunoassay (RIA) (n = 12), chemiluminescence immune assays (CLIA) (n = 4), competitive protein binding assays using rachitic rat kidney cytosol (CBA) (n = 1), enzyme-linked immunosorbent assay (ELISA) (n = 1), electrochemiluminescent immunoassay (ECLIA) (n = 1), liquid chromatography mass spectrometry (LC-MS) (n = 2), and not reported (n = 2) (Table 1). Potential control for assays was considered but was not carried out due to the limited number of high-quality studies using the same technique (RIA n = 6, CLIA n = 2, ELISA n = 1). Body composition techniques also varied, including dual-energy x-ray absorptiometry (DEXA) (n = 12), bioelectrical impedance analysis (BIA) (n = 6), skinfold thickness measurements, and equations (n = 2), and 3 studies did not report their method. Because of the variation in body composition techniques used and the limited number of high-quality studies using the same

technique (DEXA n = 6, BIA n = 1), no subgroup analysis was attempted.

2.3. Metaregression analysis

Separate unadjusted and adjusted random-effects metaregression analyses were carried out to investigate the independent contribution of the changes in the 4 main factors on the WMD of vitamin D status. A restricted maximum likelihood estimation method with backward elimination regression procedure was used in the metaregression analyses. Bubble plot was obtained with the size of the "bubble" proportional to the precision of the estimate for each of the 4 individual factors. The metaregression analyses were conducted in 3 steps: (1) unadjusted; (2) adjusted for study quality where a Jadad score was treated as a categorical variable ($0 < 3$, $1 \geq 3$); and (3) further adjustment for age, percentage of females, duration, and vitamin D in study diets (international units per day). All data analyses were carried out by Stata version 12 (2011, Stata Statistical Software: Release 12; StataCorp LP, College Station, TX, USA). $P < .05$ was considered as statistically significant.

3. Results

3.1. Systematic review

The search strategy generated 23 studies (14 RCTs and 9 single-stranded studies) whose key features are presented in Table 1. Of these studies, 12 were conducted in Europe [31-33,35-37,39,41,43,46-48]; 5, in the United States [17,34,38,40,45]; 2, in Canada [44,50]; 2, in Australia [21,22]; and 2, in the Middle East [42,49]. The study settings were all outpatient studies in a university setting or outpatient clinics. The 23 studies consisted of 2085 participants, with 74% being female, ages ranging from 12 to 62 years and study duration from 2 weeks to 2 years. All participants were overweight or obese at baseline. We found that, in 17 of 23 eligible studies, a significant increase in 25OHD was observed with a decrease in weight [17,31-33,36-39,41-48,50], a decrease in BMI in 15 studies [11,31-33,36,37,39,41,43-46,48-50], a decrease in percent fat mass (%FM) in 14 studies [17,32,33,37-41,43-47,50], and a decrease in FFM in 4 studies [32,38,44,46] (Table 1). Overall, 5 of these studies reported a significant association ($P < .05$) between 25OHD and any index of weight change [37,46-48,50], and 1 study reported a nonsignificant association [43] (Table 1).

Nine of 23 trials were assessed as high-quality studies (Jadad ≥3) and consisted of 1104 participants, with 80% being female, ages ranging from 32 to 58 years [17,21,22,31,33,34,38,41,42]. Of these 9 studies, a significant increase in 25OHD was observed with a decrease in weight in 5 studies [17,33,38,41,42], a decrease in BMI in 3 studies [17,33,41], a decrease in %FM in 4 studies [17,33,38,41], and a decrease in FFM in 1 study [38].

3.2. Metaregression analysis

Metaregression models were run unadjusted and adjusted for the effect of the confounders. However, none of the confounders was found to make a significant contribution to the change in the WMD of vitamin D when tested individually or in combination. We hence report the results obtained from the unadjusted regression analyses on (1) all weight loss studies (Table 2) and (2) adjusted for study quality (Table 3).

3.3. The effect of weight loss

The metaregression analysis for 34 arms of 17 weight loss studies included 1522 subjects and a mean age of 45 years [17,21,22,31-36,38,39,41,43-45,47,50]. The relationship favored a marginally significant increase of 6.0 nmol/L (95% CI, −12.42 to +0.47) in the WMD of 25OHD for an average weight loss of 10 kg ($P = .06$) (Table 2). When adjusting for quality of study, this association was close to significance ($P = .05$), with an increase of 6.4 nmol/L (95% CI, −12.85 to +0.12) in WMD of 25OHD for weight loss of 10 kg (Table 3).

3.4. The effect of change in %FM

The metaregression analysis for 28 arms of 13 weight loss studies included 1346 subjects, with a mean age of 44 years [17,21,22,31-33,38,39,41,43-45,50]. Results were marginally significant with an increase of 9.1 nmol/L (95% CI, −19.69 to +1.57) in the WMD of 25OHD for a 10% loss in %FM ($P = .08$) (Table 2). This result approached significance when analysis was adjusted for quality of studies where an increase of 10.5 nmol/L (95% CI, −21.87 to +0.85) in the WMD of 25OHD for a 10% decrease in %FM ($P = .06$) was observed (Table 3).

3.5. The effect of change in BMI and FFM

The metaregression analyses failed to find any significant relationship between the change in BMI and in FFM and the WMD of vitamin D in all study arms even when adjusted for quality of study (Tables 2 and 3).

4. Discussion

It is yet to be confirmed whether vitamin D is sequestered or undergoes a volumetric dilution in obesity. We questioned whether weight loss in the absence of vitamin D supplementation would increase circulating 25OHD. In this systematic review, 17 of 23 studies observed an increase in 25OHD with weight loss, but only 5 of these studies reported a significant correlation coefficient between the 2 variables [37,46-48,50]. Our metaregression analysis indicated a near significant association between weight loss and increase in 25OHD, which suggested that, for every 10 kg mean weight loss, there could be an average increase of 6.0 nmol/L in the WMD of 25OHD (Table 2; Fig. 3). When we adjusted the analysis for quality of study (with a Jadad score of ≥3 indicative of high quality), the relationship between change in weight and 25OHD was still near significance with no major change in the regression slope (Table 3). Hence, it would appear that body weight does contribute to a volumetric dilution of 25OHD [16].

After energy restriction, FM loss is a major portion of weight loss [51]. Although FFM is also lost, the precise amount

Table 1 – Human trials on weight loss and change in vitamin D status

First author, year of publication	Study details	Jadad score	Weight loss strategy	Vitamin D in food (IU/d)	Increase in vitamin D status (nmol/L)	Decrease in weight (kg)	Decrease in BMI (kg/m^2)	Decrease in FM (%)	Decrease in FFM (kg)	Assay
Ricci et al 1998 [34]	Age: 60 y Subjects: n = 30 (F) Duration: 24 wk Location: USA Study type: RCT	4	ER	NR	No change	Yes	Yes	Yes	Yes	RIA
Jensen et al 2001 [35]	Age: NR Subjects: n = 52 (F) Duration: 24 wk Location: Denmark Study type: RCT	0	ER	ER: 200 Control: 200	No change	Yes	Yes	NR	NR	CBA
Cummings 2006 [22]	Age: 53 y Subjects: n = 29 (6 M, 23 F) Duration: 12 wk Location: Australia Study type: RCT	3	ER	ER: 78 Control: 78	No change	Yes	Yes	Yes	Yes	RIA
Holecki et al 2007 [36]	Age: 50 y Subjects: n = 62 (F) Duration: 12 wk Location: Poland Study type: Single-stranded study	0	ER and PA	NR	Yes	Yes	Yes	NR	NR	RIA
Reinehr et al 2007 [37]	Age: 12 y Subjects: n = 156 (79 M, 77 F) Duration: 1 y Location: Germany Study type: Single-stranded study	0	ER and PA	ER and PA: 39	Yes	Yes, and significantly associated ($r = -0.27$; $P = .013$)	Yes	Yes	NR	CLIA
Riedt et al 2007 [38]	Age: 38 y Subjects: n = 31 (F) Duration: 24 wk Location: USA Study type: RCT	4	ER	NR	No change	Yes	NR	Yes	Yes	RIA
Hoelcki et al 2008 [39]	Age: 49 y Subjects: n = 20 (F) Duration: 12 wk Location: Poland Study type: RCT	0	ER and PA	ER and PA: NR	No change	Yes	Yes	Yes	NR	RIA
Lucey et al 2008 [31]	Age: 32 y Subjects: n = 276 (118 M, 158 F) Duration: 8 wk Location: Iceland, Spain, Ireland Study type: RCT	3	ER	ER 1: 68 ER 2: 56 ER 3: 420 Control: 64	Yes (1 group) Decrease (3 groups)	Yes	Yes	NR	NR	ELISA

Apovian et al 2009 [40]	Age: NR Subjects: n = 40 (12 M, 28 F) Duration: 12 wk Location: USA Study type: Single-stranded study	0	ER	NR	Yes	No change	No change	Yes	NR	NR
Ortega et al 2009 [32]	Age: 27 y Subjects: n = 61 (F) Duration: 2 wk Location: Spain Study type: RCT	1	ER	ER 1: 128 ER 2: 260	Yes	Yes	Yes	Yes	Yes	RIA
Chan She Ping-Delfos 2009 [21]	Age: 57 y Subjects: n = 43 (23 M, 20 F) Duration: 12 w Location: Australia Study type: RCT	3	ER, ER and PA	ER 1: 83 ER 2: 129 ER 3 and PA: 131	No change	Yes	Yes	Yes	Yes	RIA
Zinerman et al 2009 [41]	Age: 48 y Subjects: n = 83 (22 M, 61 F) Duration: 1 y Location: Germany Study type: RCT	5	ER	ER: 80	Yes	Yes	Yes	Yes	NR	RIA
Shahar et al 2010 [42]	Age: 52 y Subjects: n = 126 (M, F) Duration: 2 y Location: Israel Study type: RCT	3	ER	NR	Yes	Yes	NR	NR	NR	CLIA
Tzotzas et al 2010 [43]	Age: 40 y Subjects: n = 62 (F) Duration: 20 wk Location: Greece Study type: Single-stranded study	0	ER	NR	Yes	Yes, and associated ($r = -0.367$; $P = .065$)	Yes, and associated ($r = -0.376$; $P = .059$)	Yes	NR	ECLIA
Josse et al 2011 [44]	Age: 28 y Subjects: n = 81 (F) Duration: 16 wk Location: Canada Study type: RCT	2	ER and PA	ER and PA 1: 28 ER and PA 2: 392 ER and PA 3: 528	Yes (1 group) No change (1 group) Decrease (1 group)	Yes	Yes	Yes	Yes (2 groups) Decrease (1 group)	RIA
Mason et al 2011 [17]	Age: 58 y Subjects: n = 439 (F) Duration: 1 y Location: USA Study type: RCT	3	ER, ER and PA, PA	ER: 540 ER and PA: 538 PA: 595 Control: 447	Yes (1 group [a])	Yes	Yes	Yes	No	CLIA
Van Loan et al 2011 [45]	Age: 32 y Subjects: n = 71 (21 M, 50 F) Duration: 12 wk	2	ER	ER 1: 128 ER 2: 320	Yes (1 group) No change (1 group)	Yes	Yes	Yes	No change	RIA

(continued on next page)

Table 1 (continued)

First author, year of publication	Study details	Jadad score	Weight loss strategy	Vitamin D in food (IU/d)	Increase in vitamin D status (nmol/L)	Decrease in weight (kg)	Decrease in BMI (kg/m^2)	Decrease in FM (%)	Decrease in FFM (kg)	Assay
Christensen et al 2012 [46]	Location: USA Study type: RCT Age: 62 y Subjects: n = 175 (33 M, 142 F) Duration: 16 w	0	ER	ER: 382	Yes [a]	Yes, and significantly associated ($r = -0.21$; $P = .006$)	Yes	Yes, and significantly associated ($r = -0.16$; $P = .03$)	Yes	CLIA
Damms-Machado et al 2012 [47]	Location: Denmark Study type: Single-stranded study Age: 47 y Subjects: n = 32 (4 M, 28 F) Duration: 12 wk	0	ER	ER: 200	Yes [a]	Yes	NR	Yes, and significantly associated ($r = -0.6369$; $P < .0001$)	NR	RIA
Wamberg et al 2013 [48]	Location: Germany Study type: Single-stranded study Age: 35 y Subjects: n = 17 (9 M, 8 F) Duration: 8 and 4 wk maintenance	0	ER	NR	Yes	Yes, and significantly associated (% weight loss) ($r = 0.67$; $P = .005$)	Yes, and significantly associated ($r = -0.67$; $P = .005$)	NR	NR	LC-MS
Albadah et al 2015 [49]	Location: Denmark Study type: Single-stranded study Age: 32 y Subjects: n = 49 (M) Duration: 12 wk	0	ER	NR	Yes	NR	Yes	NR	NR	NR
Ibero-Baraibar et al 2015 [33]	Location: Saudi Arabia Study type: Single-stranded study Age: 57 y Subjects: n = 47 (24 M, 23 F) Duration: 4 wk	5	ER	ER 1: 142 ER 2: 191	Yes (1 group [a])	Yes	Yes	Yes	NR	RIA
Gangloff et al. 2015 [50]	Location: Spain Study type: RCT Age: 48 y Subjects: n = 103 (M) Duration: 1 y	0	ER and PA	NR	Yes [a]	Yes, and significantly associated ($r = -0.31$; $P < .005$)	Yes, and significantly associated ($r = -0.32$; $P < .005$)	Yes, and significantly associated ($r = -0.32$; $P < .005$)	NR	LC-MS
	Location: Canada Study type: Single-stranded study									

Age indicates mean age. $P < .05$ was considered significant.
Abbreviations: ER, energy restriction; F, female; M, male; NR, not reported; PA, physical activity.
[a] Baseline 25OHD less than 50 nmol/L; after weight loss, it was greater than 50 nmol/L.

Table 2 – Unadjusted metaregression of changes in weight and indices of body composition on vitamin D status

Outcome variable: WMD of vitamin D status	Variable	Estimated coefficient β	95% CI	P
The effect of weight loss				
Model 1	Change in weight (kg)	−0.60	−1.24, 0.04	.06
	Constant	−0.30	−4.24, 4.79	.90
The effect of decrease in BMI				
Model 2	Change in BMI (kg/m^2)	−0.13	−4.67, 4.41	.95
	Constant	2.40	−6.98, 11.78	.60
The effect of %FM loss				
Model 3	Change in FM (%)	−0.91	−1.96, 0.15	.08
	Constant	2.34	−1.22, 5.89	.18
The effect of FFM loss				
Model 4	Change in FFM (kg)	1.28	−1.25, 3.81	.30
	Constant	4.68	1.29, 8.07	.01

$P < .05$ was considered significant.

could vary with protein intake and/or increased physical activity [52], with both generally retarding loss of FFM. As both FM and FFM are major stores for the vitamin [13], we examined the effect of changes in these compartments on circulating 25OHD.

Fourteen of 23 studies observed that, with a loss in %FM, there was an increase in 25OHD [17,32,33,37-41,43-47,50] with 3 of these studies indicating a significant correlation coefficient between the 2 variables [46,47,50]. Metaregression analysis found that decreases in %FM were not significantly related to increases in 25OHD, on examination of the total data set (Table 2). When adjusted for quality of trials, this relationship between %FM loss and increase in 25OHD became marginally significant (Table 3; Fig. 4). The lack of statistical significance may have arisen not only from the smaller number of studies reporting %FM (28 study arms) and the smaller sample size (n = 1346) but also from the larger spread of effects. The β coefficient suggested that, with a 10% loss in %FM, the mean increase in 25OHD would be 10.5 nmol/L; however, the 95% CI were large at 21.8 to −0.8 nmol/L. The amount of vitamin D available in AT is approximately 103 nmol/kg [13] and represents a sizeable store in an obese person. Based on this, a 10% decrease in %FM should have resulted in a much greater increase in 25OHD than predicted by the β coefficient. There are a few potential reasons that may explain our observations.

The detection of changes in 25OHD and %FM loss would be influenced by the sensitivity of the various methods used in these trials. There is substantial interlaboratory variation in detecting 25OHD [53]. Binkley et al [53] observed that there could be up to a 2-fold difference between laboratories assaying the same sample with the same technique. Similarly, body composition techniques have limitations in their calibration, accuracy, and precision [54] dependent on the type of technique used (BIA vs DEXA vs skinfold measurement) [54,55] or within models of the same machine [56]. Dual-energy x-ray absorptiometry is considered to be the criterion standard for body composition; however, variations of up to 6% have been found between instruments from the same manufacturer [57,58]. Moreover, BIA and the skinfold technique appear to be more accurate in nonobese subjects, so there could be substantial variation in the studies of obese

Table 3 – Metaregression of changes in weight and indices of body composition on vitamin D status adjusted for study quality

Outcome variable: WMD of vitamin D status	Variable	Estimated coefficient β	95% CI	P
The effect of weight loss				
Model 1	Change in weight (kg)	−0.64	−1.28, 0.01	.05
	Jadad score (0, 1)	1.57	−2.71, 5.86	.46
	Constant	−0.84	−6.22, 4.55	.75
The effect of decrease in BMI				
Model 2	Change in BMI (kg/m^2)	−0.17	−4.77, 4.42	.93
	Jadad score (0, 1)	0.82	−4.82, 6.46	.76
	Constant	1.88	−8.28, 12.04	.70
The effect of %FM loss				
Model 3	Change in FM (%)	−1.05	−2.18, 0.08	.06
	Jadad score (0, 1)	1.43	−2.45, 5.31	.45
	Constant	1.04	−3.97, 6.05	.67
The effect of FFM loss				
Model 4	Change in FFM (kg)	2.11	−0.55, 4.77	.11
	Jadad score (0, 1)	5.30	−1.35, 11.96	.11
	Constant	1.07	−4.56, 6.69	.69

Jadad score: 0 = 0 < 3; 1 = 1 ≥ 3. $P < .05$ was considered significant.

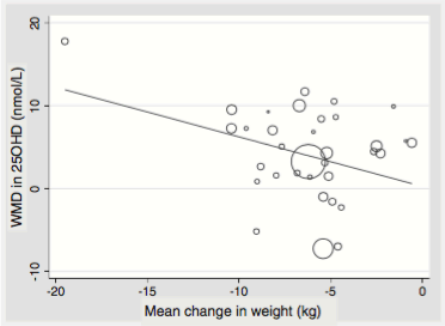

Fig. 3 – Relationship between change in weight (kilograms) and change in vitamin D status (WMD). Bubble plot of fitted metaregression line; the size of the bubbles is proportional to the precision of the estimate; WMD, weighted mean difference (nanomoles per liter) in vitamin D status.

subjects included in this review [31,32,37,39-41,43,47]. Overall, it is possible that changes in 25OHD due to fat loss and changes in %FM may not have been appropriately detected.

A second reason for the small increase in 25OHD on %FM loss is that once taken up into AT, 25OHD is released very slowly back into circulation. The latter may protect the individual from large sudden increases of a potentially toxic nutrient, while acting as a store in times of need [12]. Another further possibility is a negative feedback loop where higher circulating 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in obesity disables the production of 25OHD [59,60]. The mechanism that controls this slow release is uncertain; however, if this is true, then the shorter duration studies in this review may not detect this return of 25OHD. Importantly, vitamin D is involved in both

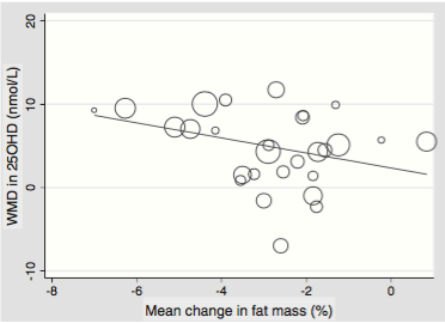

Fig. 4 – Relationship between change in FM (percentages) and change in vitamin D status (WMD). Bubble plot of fitted metaregression line; the size of the bubbles is proportional to the precision of the estimate; WMD, weighted mean difference (nanomoles per liter) in vitamin D status.

paracrine and autocrine actions in AT [61]. A nuclear VDR is expressed in AT [62], and most tissues that express VDR also contain the enzyme CYP27B1 for conversion of the circulating metabolite, 25OHD to $1,25(OH)_2D_3$. Adipose tissue has the ability to synthesize and degrade vitamin D for autocrine and paracrine use, such as in adipogenesis, lipid metabolism, and inflammation in obesity [19,63]. The expanded AT in obesity may hence engender an increased requirement of 25OHD, resulting in less return to the circulation after weight loss.

We now know that there is extensive metabolic conversion of vitamin D in storage depots. Adipose tissue is a dynamic endocrine organ, containing a variety of hydroxylase enzymes. These include the hydroxylase to convert cholecalciferol to 25OHD to $1,25(OH)_2D_3$, as well as the catabolic 24-hydroxylase for degradation of calcitrol to calcitroic acid, and 25OHD to $24,25(OH)_2D_3$ to 1-desoxycalcitroic acid, the major metabolite of 25OHD [19,48,64]. Calcitroic acid and 1-desoxycalcitroic acid are excreted through the bile into feces [65], with limited amounts found in the urine [66]. These metabolites are eliminated from the system and would not be detected in studies that only sampled the plasma compartment. In addition, there are emerging data to indicate that enzyme expression also varies with fat depots (subcutaneous vs visceral) and degrees of fatness (lean vs obese) [48]. Our current understanding is that lean and obese have similar expression of the enzyme CYP27A1 that converts 25OHD to $1,25(OH)_2D_3$ and similar expression of CYP24A1 that degrades 25OHD to calcitroic acid [64]. However, in response to weight loss, obese subjects show an elevation of the catabolic 24-hydroxylating enzyme, CYP2J2 [48], which results in several (almost 30) inactive metabolites [19,64]. Some of these include $1,24,25(OH)_3D_3$, 24-oxo, and/or 23-hydroxy groups [65] which represent pathways for vitamin D elimination [1]. Overall, many of these factors would be operative at the same time during weight loss and would go some way in explaining the smaller increase in 25OHD observed in this review. Perhaps, as Rosenstreich et al [12] opined, this slow return of 25OHD acts to protect against vitamin D toxicity that may occur if a flood of the nutrient became available during weight loss.

There are now many studies that show a positive relationship between 25OHD and muscle mass, growth, and strength/function [67-69]. This would suggest that higher vitamin D status may improve muscle mass and function, relative to those who are vitamin D insufficient. As FFM is a major store for the nutrient, we assumed that during weight loss, mobilization of the protein mass would contribute to an increase in 25OHD. However, we did not obtain a significant relationship between FFM loss and vitamin D status (Table 3). In fact, the slope of the regression line was in the opposite direction to that hypothesized (Table 3; Fig. 5). Because most studies reviewed showed a small to moderate decrease in FFM, our interpretation would be that a loss in FFM decreased circulating 25OHD. We acknowledge that this was a nonsignificant outcome but the slope was similar or greater than that obtained with %FM (Table 3; Fig. 4). Could this observation suggest that the expected positive relation between vitamin D status and muscle mass [67] is possibly bidirectional? Such a phenomenon could have negated the rise in 25OHD seen with loss of %FM (Fig. 4) and perhaps explain why the slope of weight loss change predicted was lesser than that

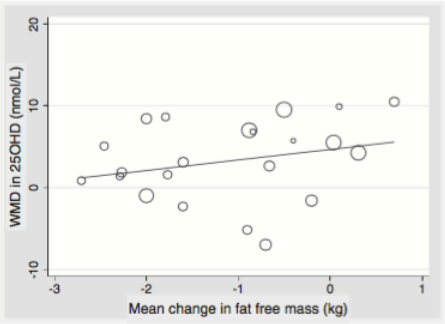

Fig. 5 – Relationship between change in FFM (kilograms) and change in vitamin D status (WMD). Bubble plot of fitted metaregression line; the size of the bubbles is proportional to the precision of the estimate; WMD, weighted mean difference (nanomoles per liter) in vitamin D status.

obtained with %FM (Table 3). It is unclear why a loss in FFM should decrease 25OHD. Fat free mass is composed of lean tissue (mainly skeletal muscle) and bone mineral content. As bone mass is also lost during energy restriction, vitamin D may prevent the expected decrease in fractional bone mass that occurs with weight loss [70]. There is good cross-talk between muscle and bone and AT and bone [70]. So, a decrease in 25OHD or a less than expected increase may also indicate diversion from plasma to other tissues during weight loss.

To the best of our knowledge, there are no other systematic reviews that have specifically addressed our question. Hence, we are unable to compare our results to the existing literature. Our search of the literature was over a long interval but covered only 2 prominent databases. We identified 23 studies, of which only 9 were quality studies with a sample size of 1104. Hence, our metaregression analysis may be limited by adequate numbers, despite contacting the authors of all studies for additional information. Seasonality of 25OHD can amount to 17 nmol/L [30,71] and have a major confounding on the stated outcomes. We were unable to control for this confounder in our analysis because season of start and completion was only mentioned in 3 studies. Moreover, there was a range of assays used in these studies as well as a range of body composition techniques that would have influenced our outcomes.

Longer duration trials of good quality (Jadad ≥3) of at least 6 months duration, which target a substantial amount of weight loss (~20% fat loss), are required. Such data would cement the clinical relevance of weight loss in normalizing vitamin D status of the obese individual. Adipose tissue is an active endocrine organ, expressing numerous receptors including vitamin D [19,72], and vitamin D is required for normal formation and function of AT [73-76]. Hence, what happens to vitamin D status before and after weight loss is important. Further investigation into vitamin D concentration in AT of obese subjects before and after weight loss may provide an insight into the amount of vitamin D and its metabolites at a cellular level. Wamberg et al [48] have provided some pioneering data in the area of vitamin D hydroxylation and catabolizing enzymes in AT. These data need validation in future trials as they provide an understanding of the dynamic influence of AT on vitamin D metabolism. Furthermore, trials on vitamin D need to report detailed body compositional changes, including body fat distribution. Dual-energy x-ray absorptiometry is now globally available and changes in android:gynoid fat can be reported, although more sophisticated determinations of subcutaneous and visceral AT changes based on computed tomography or magnetic resonance imaging scans would be worthwhile inclusions. Lastly, there is a need to report the concentrations of inactive compounds of vitamin D metabolism in both urine and serum. This would assist the evaluation of how much of the vitamin is unavailable for metabolism during weight loss.

In conclusion, this systematic review provides good evidence for an inverse relationship between weight and fat loss and 25OHD in obesity. Although overall in support of a volumetric dilution phenomenon in obesity, the review cannot discount a sequestration effect and possibly extensive degradation of 25OHD after weight loss.

PKP conducted the literature search, assembled the tables, extracted the data, and cowrote the manuscript. MJS generated the idea, conceptualized the review process, cross-checked data extraction, and co-wrote the manuscript. YZ prepared the data extraction template, conducted the statistical analysis, and cowrote the manuscript. All authors contributed to the writing of the final manuscript.

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Appendix C The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor Survey

Public Health Nutrition: page 1 of 12 doi:10.1017/S1368980016001609

The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor survey

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Abstract

Objective: To examine the associations between serum 25-hydroxyvitamin D (25(OH)D), dietary Ca intake and presence of the metabolic syndrome (MetS).

Design: A stratified cluster sample of a population aged 18–75 years from the Victorian Health Monitor survey.

Setting: Non-institutionalized adults living in private dwellings in Victoria, Australia.

Subjects: Adults (n 3404) with complete data and without type 1 or type 2 diabetes.

Results: Adjusted for sociodemographic factors, physical characteristics and dietary covariates including Ca intake, every 10 nmol/l increase in serum 25(OH)D was significantly associated with decreased odds of MetS (adjusted odds ratio (AOR) = 0·85, 95 % CI 0·80, 0·89; $P < 0·001$). Relative to the low 25(OH)D tertile (median 33 nmol/l), there was a progressive decrease in odds of MetS that reached significance with the high 25(OH)D tertile (median 77 nmol/l; AOR = 0·35, 95 % CI 0·26, 0·48; $P < 0·001$). Every 500 mg/d increase in Ca intake adjusted for 25(OH)D did not reduce odds of MetS (AOR = 0·81, 95 % CI 0·66, 1·06; $P = 0·141$) but approached significance if unadjusted for 25(OH)D in the final model (AOR = 0·81, 95 % CI 0·64, 1·02; $P = 0·073$). No significant effect was obtained for tertiles of Ca intake. However, Ca and vitamin D tertile combinations suggested a beneficial effect of high Ca (median 1233 mg/d) only at low and medium 25(OH)D. The high 25(OH)D tertile was associated with significantly decreased odds of MetS regardless of Ca intake.

Conclusions: A high vitamin D status significantly reduced the odds of MetS. A high Ca intake may have a similar favourable outcome but only at lower circulating concentrations of 25(OH)D.

Keywords
Metabolic syndrome
Vitamin D
Calcium
25-Hydroxyvitamin D

Vitamin D is a secosteroid that is produced cutaneously through solar UV-B irradiation of 7-dehydrocholesterol present in the skin[1,2]. The second source of vitamin D is via food intake and like for Ca, the greatest contribution to intake comes from milk and other dairy products. Vitamin D undergoes two hydroxylation steps, one in the liver and one in the kidney. The final hydroxylation step in the kidney converts 25-hydroxyvitamin D (25(OH)D) to its active metabolite, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), and the enzyme 1-α-hydroxylase catalyses this conversion[3]. Interestingly, expression of the nuclear vitamin D receptor (nVDR) and 1-α-hydroxylase is present not only in the kidneys but also many other tissues of the body[4], including the pancreas[2] and immune cells[5–8]. Thus many tissues have the ability to locally synthesize $1,25(OH)_2D$ from 25(OH)D and the potential to contribute to circulating concentrations[4]. The active metabolite can then bind to the nVDR, where it forms a heterodimer with the retinoid X receptor[2]. It is now recognized that nVDR regulates approximately 3 % of the human genome (~700 genes)[2,5] and, together with its wide distribution, this provides some foundation for the study of extra-skeletal benefits of vitamin D.

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Public Health Nutrition

The metabolic syndrome (MetS) is a clustering of risk factors that greatly increases the risk of CVD and type 2 diabetes mellitus (T2DM). Insulin resistance (IR) is a key player in the development of MetS; however, factors other than IR are also important. Clinical diagnosis of MetS is based on the presence of three or more of the following markers of chronic disease: (i) greater waist circumference; (ii) raised fasting plasma glucose; (iii) hypertension (elevated systolic blood pressure or diastolic blood pressure); and (iv) dyslipidaemia (raised TAG and low HDL cholesterol)[9]. The prevalence of MetS in Australia is high with ~30% of adults classified as having the syndrome[10]; a figure comparable to that in other developed countries[11,12]. For a sun-drenched country with abundant milk supplies, it is surprising that both vitamin D insufficiency and low Ca intake are highly prevalent in Australia[13–15]. Inadequate vitamin D status has been implicated as a causal factor in many chronic conditions[16] including T2DM[17], MetS[18,19], CVD[20], hypertension[21,22] and IR[23,24]. We have previously discussed the potential for Ca and vitamin D to regulate body weight[25] and influence the risk of chronic disease[26–29]. Documented pathways include Ca's stimulation of fat oxidation, heightened diet-induced thermogenesis, increased faecal fat excretion[27], reduced circulating TAG[30] and the potential for vitamin D to increase resting metabolism[31]. Emerging data also support a beneficial effect on IR and T2DM[27,32–34]. However, a consensus document produced by the Institute of Medicine found little convincing evidence available at the time in support of extra-skeletal effects of vitamin D[35]. The aim of the present study was to investigate whether there was a link between population-based measures of vitamin D status, dietary Ca intake and the prevalence of MetS.

Methods

To fulfil our objectives we used a state-wide representative survey of Victorian adults: the Victorian Health Monitor (VHM)[36]. The VHM was conducted between May 2009 and April 2010. A stratified cluster sample was selected, based on census collection districts within the eight Victorian Government Department of Health regions. Fifty randomly selected census collection districts were included in the sample, twenty-five from metropolitan and twenty-five from rural Victoria. One eligible person (aged 18–75 years) from each household in each census collection district was randomly selected to participate. The VHM was approved by the Human Research Ethics Committee (HREC) of the Baker IDI Heart and Diabetes Institute, Melbourne, Victoria[37]. The analysis of the VHM database was also approved by the HREC at Curtin University (HREC approval number: SPH-19-2014).

The VHM involved an initial household visit to participants to collect demographic information, followed by a participant visit to a local test site to collect risk factor information and undergo biomedical and physical examination. Participants were then asked to complete three 24 h dietary recall interviews, which were conducted over a 6-week period. The overall response rate for the VHM was 38% and a final sample of 3653 participants was achieved.

The response rate in the VHM survey is comparable to similar Australian surveys including the Australian Health Survey: Biomedical Results 2011–12 (response rate 37·1%)[38] and the Australian Diabetes, Obseity and Lifestyle Study (response rate 37%)[39]. To identify any potential selection bias in the VHM between participants and non-participants, key demographic characteristics were compared. A minimal level of difference was found between the two groups[37]. Demographic characteristics of participants of the VHM survey were also similar to those from the annual Victorian Population Health Surveys conducted in 2010 (n 7535) and 2011–12 (n 33 673) by the Victorian Government, which had response rates of 73%[40] and 67%[41], respectively. This would suggest that the level of bias in the VHM is probably no different from that in the larger Victorian Population Health Survey.

Test sites for the collection of biomedical and physical measures were set up specifically for the purposes of the study in census collection districts included in the sample. The procedures used for the biomedical examination were closely aligned with the protocol recommended by the WHO[42]. Participants provided written informed consent upon arrival at test sites and were asked to stay until all tests were complete. Abnormal test results were reviewed by a study doctor who determined whether a result warranted follow-up with a participant. Further details on the survey protocols and procedures can be found in the VHM report[36] and the VHM food and nutrition report[43].

Sample

In the present study, we excluded: (i) participants with missing glycosylated Hb (HbA1c) data (n 31); (ii) those with HbA1c ≥ 6·7% as they were classified as having T2DM according to the American Diabetes Association cut-offs (n 39)[44]; (iii) those with diagnosed T2DM (n 140); (iv) those with type 1 diabetes (n 9); (v) participants on diabetic medications (n 25); and (vi) those with missing metabolic components for MetS diagnosis (n 5). Hence, a total of 3404 participants were included in the analysis. Information on the use of supplements (Ca or vitamin D) was not available in this survey.

Assessment of vitamin D status

Blood samples were collected via venepuncture after an overnight fast of 10 h or more. Blood was immediately transported to an accredited central laboratory in Melbourne, Australia. The measurement of serum 25(OH)D concentration was based on the DiaSorin Corporation

Liaison® 25(OH)D total assay. The assay is an automated, direct competitive chemiluminescent immunoassay that measures ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) to provide a total value for circulating vitamin D in nmol/l. The detection limit was 10 nmol/l. The ALTM (All Laboratory Trimmed Mean) was not computed by the laboratory, nor were results compared with a TV ('Target Value') assigned by the NIST (National Institute of Standards and Technology) Reference Measurement Procedure.

Assessment of dietary calcium intake

Dietary intake data were obtained by multiple-pass 24 h diet recall using computer-assisted telephone interviews. The first diet recall interview was conducted within 5 to 7 d of the participants attending the biomedical examination. Two subsequent diet recall interviews were conducted at 2-week intervals following the first diet recall interview. A total of 3653 participants attended and participated in survey components at test sites. Three dietary recalls were conducted, with a total of 10 307 dietary recalls completed, where 96 % completed one dietary recall, 94 % completed two dietary recalls and 92 % completed three dietary recalls. Details of the dietary recall and post-interview processing methodology employed are described in the VHM food and nutrition report[43].

All dietary recall interviews were conducted by certified dietitians from the Department of Nutrition and Dietetics, Monash University. Interviewers were trained to assure competency and consistency in collected dietary recall information. Interviewers used a food model book to aid participants with their description of portion sizes of the foods and beverages they had consumed. The food model book prompted dietary recall by including frequently forgotten foods and eating occasions, and assisted with portion size estimation with 'to scale' photographs of food and beverage containers, measuring spoons and cups[43].

The FoodWorks® nutrition software (FoodWorks® Interview) was employed for implementation of dietary recalls. The dietary recall used a multiple-pass approach to assist participants to sufficiently recall their food and beverage intakes. The software includes a scripted guide for interviewers to help prompt participants for food recall in each interview. Interviewers were able to interrupt and prompt for further details on food items if required. Further information on the multiple-pass dietary recall process has been described in detail in the VHM food and nutrition report[43].

On completion of the interviews, volume conversion factors were developed to convert food volumes into food weights. Conversions of food volumes to weights were done by 'reference to published data, by measuring the weight and volume of specific foods, or by considering the food as very similar to another food for which a volume conversion factor was already available'[43]. The AUSNUT 2007[45] nutrient composition data were used to calculate nutrient intakes based on estimated food intakes. The mean intake for each nutrient was computed for each participant based on information collected from three 24 h dietary recalls and was used in the analysis. This information was used to get a single measure of nutrient intake for each participant[43].

Physical activity level

The following criteria were used to define each participant's level of physical activity: (i) sufficiently physically active (≥150 min of 'physical activity time' per week); (ii) insufficiently physically active (1–149 min of 'physical activity time' per week); and (iii) physically inactive (0 min of 'physical activity time' per week)[36]. 'Physical activity time' was calculated as the sum of the time spent walking or performing moderate activity plus double the time spent in vigorous physical activity (to reflect its greater intensity)[46].

Anthropometric measurements

Anthropometric measurement methods for weight, height and waist circumference have been previously described in the VHM report[36].

Biomedical measurements

Blood collection was conducted via venepuncture after an overnight fast of 10 h or more. Blood samples were assessed for the following factors: total cholesterol, HDL cholesterol, TAG, Hba1c and fasting plasma glucose levels. Blood samples were centrifuged on site and were analysed at a separate central laboratory on a Siemens ADVIA 2400 Clinical Chemistry System. Blood components were measured as following: total cholesterol using enzymatic (oxidase/peroxidase) methods; HDL cholesterol using the elimination/catalase method; TAG using the GPO Trinder reagent set with serum blank; blood glucose using the hexokinase method; and HbA1c was measured by immunoassay (Roche Integra chemistry analyser)[36].

Blood pressure measurements

Sitting blood pressure measurements (GE Dinamap 8100 Vital Signs Monitor) were performed in triplicate on each participant, after a rest period of 5 min. The average of the two closest measurements (<10 mmHg for systolic blood pressure and <6 mmHg for diastolic blood pressure) were used in the analysis. Further details have been presented in the VHM report[36].

Classification of metabolic syndrome

MetS was classified according to the criteria from the joint interim statement of several major organizations[9]. Individuals were classified as having MetS if they had

three or more of the following five components: (i) elevated TAG ≥1·7 mmol/l (≥150 mg/dl); (ii) reduced HDL-C <1·0 mmol/l (<40 mg/dl) in males and <1·3 mmol/l (<50 mg/dl) in females, or on lipid-lowering therapy; (iii) hypertension (systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg), or on anti-hypertensive medications; (iv) elevated fasting plasma glucose ≥5·6 to 6·9 mmol/l (≥100 to 124 mg/dl) but free of diabetes; and (v) elevated waist circumference ≥94 cm for males or ≥90 cm for Aboriginal and Torres Strait Islander, Asian and South American males and ≥80 cm for females. In the current analysis participants were categorized into having or not having MetS (yes/no).

Statistical analysis

The main outcome variable was the status of MetS (yes/no). The primary exploratory variables of interest were serum 25(OH)D concentration and Ca intake, which were both categorized into tertiles: low 25(OH)D (range 10–44 nmol/l; median 33 nmol/l), medium 25(OH)D (range 45–65 nmol/l; median 54 nmol/l) and high 25(OH)D (range 65–204 nmol/l; median 77 nmol/l); and low Ca (range 72–719 mg/d; median 579 mg/d), medium Ca (range 720–1009 mg/d; median 858 mg/d) and high Ca (range 1010–3726 mg/d; median 1233 mg/d). The association between all possible combinations of serum 25(OH)D concentration and Ca intake tertiles (thereby nine levels in total) and MetS was examined in the present study, with mutual adjustment for the other components. Serum 25(OH)D concentration was also tested as a continuous variable for every 10 nmol/l increment, while Ca intake was tested as a continuous variable for every 500 mg/d increment.

In the first stage of the analysis, demographic statistics and differences between the serum 25(OH)D concentration and Ca intake tertiles were tested by the independent-samples *t* test and frequency tabulation. Furthermore, to investigate the effect of the categorical predictors of interest on the risk of having MetS and higher value of its components, a χ^2 test and simple binary logistic regression analysis were then conducted to obtain the crude unadjusted odds ratios and corresponding 95% confidence intervals.

Multiple logistic regression analysis was then carried out to calculate the adjusted odds ratios (AOR) and 95% confidence intervals for the relationships between serum 25(OH)D concentration or Ca intake and having MetS. Analyses were conducted using the statistical software package IBM SPSS Statistics for Windows, Version 21.0. Complex samples analysis was applied to adjust for the unequal selection probability due to the multistage stratified cluster-sampling procedure used in the VHM survey. Appropriate clustering and weighting variables were used to compute appropriate standard errors and confidence intervals in the complex samples analysis procedure. A *P* value of less than 0·05 was accepted as statistical significance.

Confounders

In our analysis we considered and tested several risk modifiers, based on our experience[47] and that of others[18,48–50]. Accordingly we included the following demographic factors: weight, age, gender, country of birth, income, education level, physical activity level, smoking status and season. Dietary factors included intakes of: alcohol, dietary fibre, energy, Mg, retinol, 25(OH)D concentration (Ca intake model only) and Ca intake (25(OH)D concentration model only). Age, weight, alcohol, dietary fibre, energy intake, Mg, retinol, 25(OH)D concentration and Ca intake were entered into the regression model as continuous variables. Country of birth was identified according to those born in Australia and those born overseas. Education level was categorized according to three levels: tertiary education, TAFE/diploma/certificate and high school or less. Smoking status was assessed on the basis of three categories: current smoker, ex-smoker and non-smoker. Income levels were categorized according to four categories: ≥$AU 70 000, $AU 30 001–70 000, <$AU 30 000 and don't know/refused. Season of biomedical examination was categorized as summer, autumn, winter and spring.

Rationale of analysis

In the current analysis we examined the relationship of serum 25(OH)D concentration and Ca intake on MetS through a series of questions that resulted in different models:

1. What was the unadjusted relationship between 25(OH)D and Ca intake with MetS? (crude model).
2. What was the confounding influence of sociodemographic factors on the relationship of 25(OH)D/Ca intake with MetS? (model 1).
3. What was the potential influence of dietary factors on the relationship of 25(OH)D/Ca intake with MetS? (model 2).

Results

The present study population consisted of a total of 3404 adults with a mean age of 49 years. The overall prevalence of MetS was 21·6%, with a larger proportion of males (22%) having MetS than females (14%; $P<0·001$). The mean serum 25(OH)D concentration of those with MetS was 49·6 nmol/l, significantly lower than that of participants without MetS which was 57·5 mmol/l ($P<0·001$). The mean dietary Ca intake was 849 mg/d in those with MetS and 926 mg/d in those without MetS ($P<0·001$; Table 1).

Table 1 Demographic and clinical characteristics by the presence/absence of metabolic syndrome (MetS) among non-diabetic adults (*n* 3404) aged 18–75 years from the Victorian Health Monitor survey, May 2009–April 2010

	Absence of MetS (*n* 2669)				Presence of MetS (*n* 735)				
Characteristic	*n*	%	Mean	SE	*n*	%	Mean	SE	*P* value
Age (years)			41	0·9			52	1·0	<0·001
Weight (kg)			75·7	0·5			91·6	0·8	<0·001
Gender									<0·001
Male	1237	78		2·0	344	22		2·0	
Female	1565	86		1·1	257	14		1·1	
Country of birth									0·541
Born in Australia	2139	82		1·4	454	18		1·4	
Born overseas	658	81		2·0	153	19		2·0	
Education level									<0·001
Tertiary education	1136	87		1·5	170	13		1·5	
TAFE/diploma/certificate	580	80		2·3	140	20		2·3	
High school or less	1075	78		1·7	303	22		1·7	
Income									0·098
≥$AU 70 001	1341	84		1·4	250	16		1·4	
$AU 30 001–70 000	837	79		2·1	220	21		2·1	
<$AU 30 000	456	81		2·6	110	19		2·6	
Don't know/refused	157	82		3·8	33	18		3·8	
Physical activity level									0·011
Sufficient physical activity (≥150 min/week)	1929	83		1·5	384	17		1·5	
Insufficient physical activity (<149 min/week)	704	81		1·9	168	19		1·9	
Inactive (0 min/week)	153	71		4·8	62	29			
Smoking status									<0·001
Current smoker	423	80		2·5	109	20		2·5	
Ex-smoker	694	75		2·1	224	25		2·1	
Non-smoker	1669	85		1·4	283	15		1·4	
Season of biomedical examination									0·182
Summer	155	75		5·2	52	25		5·2	
Autumn	677	84		2·0	131	16		2·0	
Winter	964	84		1·8	182	16		1·8	
Spring	991	80		2·8	252	20		2·8	
Vitamin D status									
Serum 25(OH)D (nmol/l)			57·5	2·1			49·6	2·1	<0·001
25(OH)D tertile									<0·001
Low 25(OH)D (33 nmol/l)†	857	77		2·1	252	23		2·1	
Medium 25(OH)D (54 nmol/l)†	925	80		2·3	237	21		2·3	
High 25(OH)D (77 nmol/l)†	1013	89		1·3	120	11		1·3	
Dietary variables									
Dietary Ca intake (mg/d)			926·0	11·4			849·0	20·0	<0·001
Ca tertile									0·001
Low Ca intake (579 mg/d)†	847	77		1·8	245	23		1·8	
Medium Ca intake (858 mg/d)†	908	83		1·4	187	17		1·4	
High Ca intake (1233 mg/d)†	924	85		1·5	163	15		1·5	
Total energy intake (kJ/d)			9768	134			9442	163	0·021
Alcohol (g/d)			12·3	0·7			15·3	0·9	<0·001
Dietary fibre (g/d)			26·5	0·4			25·3	0·5	0·006
Mg (mg/d)			418·9	6·6			397·2	7·6	0·008
Retinol (μg/d)			433·8	23·2			454·5	56·1	0·428
Metabolic components									
Waist circumference (cm)			86·4	0·7			102·5	0·7	<0·001
Fasting plasma glucose (mmol/l)			4·9	0·02			5·5	0·04	<0·001
HDL cholesterol (mmol/l)			1·5	0·02			1·2	0·02	<0·001
TAG (mmol/l)			1·1	0·02			2·1	0·06	<0·001
Systolic blood pressure (mmHg)			122	0·7			136	0·9	<0·001
Diastolic blood pressure (mmHg)			71	0·5			81	0·4	<0·001

Data are presented as mean estimate (weighted) % for categorical variables, and mean estimate (weighted) and SE for continuous variables. Differences in the continuous and categorical variables between groups were assessed by the independent-samples *t* test and the χ^2 test, respectively.
†Median of the tertile group.

Association between tertiles of serum 25-hydroxyvitamin D concentration, calcium intake and presence of metabolic syndrome

Every 10 nmol/l increment in serum 25(OH)D concentration reduced the likelihood of having MetS by 15% (model 2; Table 2). The crude model indicated that those in the highest tertile of serum 25(OH)D concentration had a 60% lower odds of having MetS. After adjusting for sociodemographic variables (model 1), the significant inverse association between serum 25(OH)D

Table 2 Odds ratio of having metabolic syndrome by tertiles of serum 25-hydroxyvitamin D (25(OH)D) concentration among non-diabetic adults (*n* 3404) aged 18–75 years from the Victorian Health Monitor survey, May 2009–April 2010

	Crude model		Model 1		Model 2	
	COR	95 % CI	AOR	95 % CI	AOR	95 % CI
25(OH)D, continuous (10 nmol/l)	0·87	0·82, 0·92	0·82	0·78, 0·85	0·85	0·80, 0·89
P value		<0·001		<0·001		<0·001
25(OH)D tertile						
Low 25(OH)D (33 nmol/l)†		Ref.		Ref.		Ref.
Medium 25(OH)D (54 nmol/l)†	0·87	0·66, 1·14	0·69*	0·52, 0·90	0·77	0·58, 1·04
High 25(OH)D (77 nmol/l)†	0·40*	0·29, 0·56	0·29*	0·22, 0·38	0·35*	0·26, 0·48
P value for trend		<0·001		<0·001		<0·001

COR, crude odds ratio; AOR, adjusted odds ratio; Ref., lowest 25(OH)D tertile served as the reference group.
Model 1: adjusted for age, gender, country of birth, income, education, smoking and season.
Model 2: adjusted for model 1 covariates plus energy intake, physical activity level, body weight, alcohol, dietary fibre, Mg, Ca and retinol.
*Significant in comparison to the reference group at 5 % significance level.
†Median of the tertile group.

Table 3 Odds ratio of having metabolic syndrome by tertiles of dietary calcium intake among non-diabetic adults (*n* 3404) aged 18–75 years from the Victorian Health Monitor survey, May 2009–April 2010

	Crude OR		Model 1		Model 2	
	COR	95 % CI	AOR	95 % CI	AOR	95 % CI
Ca intake, continuous (500 mg/d)	0·74	0·62, 0·89	0·75	0·61, 0·91	0·81	0·66, 1·06
P value		0·002		0·004		0·141
Ca tertile						
Low Ca (579 mg/d)†		Ref.		Ref.		Ref.
Medium Ca (858 mg/d)†	0·71*	0·56, 0·90	0·73*	0·56, 0·96	0·92	0·63, 1·33
High Ca (1233 mg/d)†	0·61*	0·46, 0·81	0·63*	0·46, 0·86	0·83	0·56, 1·21
P value for trend		0·002		0·012		0·613

COR, crude odds ratio; AOR, adjusted odds ratio; Ref., lowest Ca tertile served as the reference group.
Model 1: adjusted for age, gender, country of birth, income, education, smoking and season.
Model 2: adjusted for model 1 covariates plus energy intake, physical activity level, body weight, alcohol, dietary fibre, Mg and 25-hydroxyvitamin D concentration.
*Significant in comparison to reference group at 5 % significance level.
†Median of the tertile group.

concentration and presence of MetS remained. After adjustment for dietary variables (alcohol, dietary fibre, energy, Mg, Ca and retinol), participants in the highest 25(OH)D tertile had a 65 % lower odds of having MetS compared with those in the lowest 25(OH)D tertile (model 2; Table 2).

Table 3 shows that every 500 mg/d increment in dietary Ca intake reduced the likelihood of having MetS by 25 % after adjusting for sociodemographic variables in model 1, but the reduction became non-significant after adding dietary variables (alcohol, dietary fibre, energy, Mg and serum 25(OH)D concentration) in model 2. If we did not control for serum 25(OH)D in the latter model, the AOR approached significance (AOR = 0·81, 95 % CI 0·64, 1·02; $P = 0·073$) but was non-significant on controlling for 25(OH)D (Table 3, model 2; $P = 0·141$). Those in the highest tertile of dietary Ca intake had significantly reduced odds of having MetS by 39 % in the crude model and 37 % in model 1 in comparison with those in the lowest tertile of dietary Ca intake; however, the comparison was not significant when dietary factors were added to model 2 (Table 3). Based on previous evidence we tested for potential interactions between serum 25(OH)D concentration, Ca intake and age, gender, smoking status, physical activity, county of birth and education level; however, no significant interactive effects were found[15]. Furthermore, interactions between serum 25(OH)D concentration, Ca and dietary variables (alcohol, dietary fibre, energy, Mg and retinol) were tested but none were significant.

Association between combined effects of serum 25-hydroxyvitamin D concentration and calcium intake and presence of metabolic syndrome

In view of finding no significant interaction between serum 25(OH)D status and Ca intake ($P = 0·651$), the regression analysis was extended to examine the effect of combining serum 25(OH)D concentration and Ca intake tertiles on MetS (Fig. 1). The combination of low serum 25(OH)D tertile (median 33 nmol/l) and low Ca intake tertile (median 579 mg/d) was the reference group. After controlling for confounding factors, the combination of high serum 25(OH)D and low, medium or high Ca intake significantly reduced the odds of having MetS by 72, 70 and 66 %, respectively (Fig. 1).

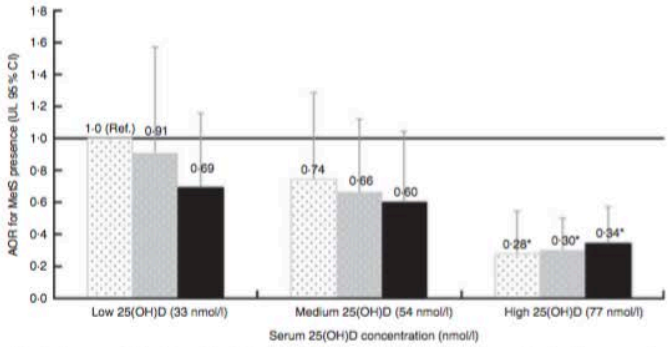

Fig. 1 Combined effects of serum 25-hydroxyvitamin D (25(OH)D) concentration (median of the tertile group given in parentheses) and dietary calcium intake (□, low calcium, median 579 mg/d; ▨, medium calcium, median 858 mg/d; ■, high calcium, median 1233 mg/d) on the presence of metabolic syndrome (MetS) among non-diabetic adults (*n* 3404) aged 18–75 years from the Victorian Health Monitor survey, May 2009–April 2010. Adjusted odds ratios (AOR), with the upper limit (UL) of the 95 % confidence interval represented by vertical bars, adjusted for age, gender, country of birth, income, education, smoking, season, physical activity level, weight, alcohol, dietary fibre, magnesium, retinol and energy intake. 'Ref.' indicates that the lowest 25(OH)D and lowest calcium tertile served as the reference group; *significant in comparison to reference group at 5 % significance level

Discussion

We investigated the individual and combined association of serum 25(OH)D concentration with dietary Ca intake on MetS. In addition to many confounders, we controlled for Ca intake in the 25(OH)D model and for 25(OH)D concentration in the Ca model, to investigate their effect, independent of each other. The results of this representative sample of adults from an Australian state have indicated that higher serum 25(OH)D concentration *per se* was associated with significantly reduced odds of MetS (Table 2). However, this was not statistically significant for every model of Ca intake tested (Table 3). As a continuous variable the overall pattern for Ca was in the same direction as 25(OH)D and with a lower AOR (Table 3). If we did not control for 25(OH)D in the Ca intake continuous model, the AOR approached significance (AOR = 0·81, 95 % CI 0·64, 1·02; $P = 0·073$) but on controlling for 25(OH)D (Table 3, model 2; $P = 0·141$), this was non-significant. Such outcomes suggest that prevailing serum 25(OH)D concentrations could modulate the potential effect of Ca on MetS.

Our findings are consistent with other cross-sectional and prospective studies where an inverse association between 25(OH)D concentration, Ca intake and MetS was observed[18,19,48,51–55]. One cross-sectional study found a 67 % reduction in the odds of having MetS among those in the highest 25(OH)D tertile (68–231 nmol/l) *v.* the lowest tertile (9–45 nmol/l)[48]. Our study obtained relatively similar results where the highest tertile of 25(OH)D was found to contribute a 65 % reduced odds for MetS in comparison to the lowest tertile (Table 2). The study by Hypponen *et al.*[48] had double the sample size but adjusted only for gender, month and hour of blood measurement. In comparison we controlled for additional sociodemographic, anthropometric and dietary covariates. A more recent prospective study in the elderly also found an inverse association between MetS and high 25(OH)D (≥75 nmol/l), although the magnitude of their findings was much lower[53]. Furthermore, a large prospective study reflected our results and found a 36 % reduction in odds of having MetS in the highest Ca intake group (1005–2596 mg/d) in comparison to the lowest Ca group. Overall, despite differences between such studies in sample sizes, study design (cross-sectional *v.* prospective), age of subjects and confounders used, the protective effect of vitamin D in reducing the odds of having MetS appears consistent.

We also examined the potential additive effects of tertile combinations of serum 25(OH)D concentration and Ca intake on MetS (Fig. 1). The outcomes were interesting since they suggested that at low and medium tertiles of 25(OH)D, there was a trend for increasing Ca intake to reduce AOR of MetS (Fig. 1). However, in the highest 25(OH)D tertile this trend disappeared, with significantly reduced AOR across the range from low to high Ca intakes. This was suggestive of a plateau effect, raising the possibility of a threshold to the interplay between Ca and 25(OH)D on functional outcomes.

It is now well known that increasing Ca intake increases passive Ca absorption from the gastrointestinal tract[54]. A higher Ca intake also increases the half-life of 25(OH)D in circulation[55] and together these actions may explain the effect of high Ca in the lowest 25(OH)D tertile (Fig. 1). However, a key physiological function of 25(OH)D is the maintenance of Ca homeostasis via active intestinal Ca

absorption[54,56,57]. So an improvement in vitamin D status from the low to medium tertile (Fig. 1) would further increase active Ca absorption and possibly allow for a greater effect of Ca on MetS. In support of such a paradigm was the observation that the overall effect of Ca in the medium 25(OH)D tertile was stronger than in the low 25(OH)D tertile (Fig. 1). While 25(OH)D and Ca absorption have a positive relationship, there is a plateau to this effect. Above ~80 nmol/l, active Ca absorption does not respond to further increases in 25(OH)D[54]. It is notable that the latter concentration falls within the highest tertile of 25(OH)D in the present study and may explain why increasing Ca intake ceases to have any added benefit in the highest tertile (Fig. 1).

There is another related and important facet to these relationships. A raised parathyroid hormone concentration is associated with an increased risk of MetS[29,58,59]. Increases in dietary Ca and in serum 25(OH)D would lower circulating parathyroid hormone. Recent data have described the exponential decline in parathyroid hormone with increases in 25(OH)D[60]. The analysis indicated two inflection points in the relationship, with the second plateau at 25(OH)D concentrations above ~70 nmol/l where parathyroid hormone was maximally suppressed[60]. We acknowledge that this threshold value of 25(OH)D is not universally accepted[61] and that further work is necessary. However, it serves the argument that, at the highest tertile of 25(OH)D in the present study, the negative effects of a raised parathyroid hormone level on MetS could be significantly diminished relative to the previous tertiles. Overall, our results argue that Ca intake has an added effect with 25(OH)D on reducing MetS, but this applies only up to the medium tertile of 25(OH)D (Fig. 1). Above the latter the observed effects are due mainly to 25(OH)D *per se*. There is some evidence in the literature in support of threshold effects, especially for outcomes that impinge on MetS. A randomized controlled trial has demonstrated that following vitamin D supplementation, significant increases in insulin sensitivity (HOMA%S) were observed only in those who achieved a 25(OH)D concentration of 80 nmol/l and had maintained that value for 6 months[24]. In a weight-loss randomized controlled trial, participants who achieved 80 nmol/l at 12 months demonstrated significantly greater losses in weight, percentage fat mass and waist circumference, compared with those who did not[62]. We cannot predict the threshold value of 25(OH)D from our study. Moreover, as the outcomes of these randomized controlled trials were derived from *post hoc* analyses, they only support the hypothesis rather than validate an 80 nmol/l cut-off.

Potential mechanisms

There are many mechanistic pathways to support our observations of a protective effect of 25(OH)D concentrations on MetS. An animal study suggests an independent effect of 25(OH)D on β cells, with improvements in impaired glucose tolerance and insulin secretion, despite prevailing plasma Ca concentrations[63]. $1,25(OH)_2D$ has a role in insulin secretion[64], where it stimulates the expression of the insulin receptor and increases the responsiveness to glucose transport. During vitamin D deficiency β-cell function is inhibited, leading to a decrease in insulin secretion[65]. In addition, inadequate 25(OH)D concentration is associated with IR[66–68]. While we acknowledge that IR does not always explain all of MetS[69–71], it is a key feature in the pathophysiology of the syndrome[72]. The nVDR and 1-α-hydroxylase enzyme are found in tissues not related Ca metabolism, such as in cardiac myocytes, endothelial and smooth vascular muscle cells[70]; potentially underscoring a role of 25(OH)D in cardiovascular health. The renin–angiotensin system is important in the regulation of blood pressure[73] and low 25(OH)D concentration may dysregulate control of the renin–angiotensin system[71]. In this context lower 25(OH)D concentration has been found to be inversely correlated with measures of arterial stiffness and also to increased arterial resistance, hypertension and endothelial dysfunction[74–77]. Moreover higher vitamin D status could also reduce islet β-cell damage by reducing islet renin–angiotensin system activity, thereby reducing the risk of hyperglycaemia[78].

The beneficial effect of Ca on features of MetS may arise from both its absorbed fraction and its unabsorbed fraction in the gastrointestinal tract[27]. There is now increasing evidence that Ca intake may influence fat balance and hence energy balance. Dietary Ca increases whole-body fat oxidation and this could, potentially, reduce circulating fatty acids/lipids[27,79]. Unabsorbed Ca is not without metabolic effects[27]. A meta-analysis indicates that for dairy Ca intake of ~1200 mg/d, an increase of ~5 g/d in faecal fat can be expected[80]. This arises from the interaction of non-absorbed Ca and dietary fat in the gastrointestinal lumen, leading to Ca–fatty acid soap formation and hence its eventual excretion. These outcomes may contribute to lower circulating TAG and other lipid fractions seen with Ca supplementation[30]. Finally, as with other chronic non-communicable conditions, MetS is a low-grade chronic inflammatory state. We, and others, are of the opinion that adequate vitamin D has a significant role in ameliorating the inflammatory state in chronic disease[8,81,82].

Study limitations

The cross-sectional design has permitted only an examination of associations between Ca intake, vitamin D status and MetS. Although we have controlled for recognized confounders, we cannot establish which came first, lower 25(OH)D concentration and Ca intake or having MetS. An increased requirement for these nutrients in chronic conditions like MetS is a possibility and may account for a

reverse causation. Unlike some European countries, there is no mandatory fortification of the Australian food supply for these nutrients. Unfortunately the VHM survey did not include information on Ca and vitamin D supplement usage. Such information would have potentially allowed us to tease out the effect of food-derived Ca and sunlight-derived vitamin D status (since vitamin D in Australian foods is low) *v.* pharmacological intake. However, we approached the potential confounding effect of supplement Ca intake by using random generated surrogate data for different age groups, based on the Ca supplement intake percentages collected in the Australian Health Survey 2011–12[85]. We found that the change between crude and adjusted effect estimates was much less than 10 %; a cut-off criterion for being a sizeable confounder in epidemiology research[84]. Hence, we do not anticipate significant confounding by supplement-derived Ca intake on the association between dietary Ca intake and the risk of MetS in the current study.

Serum 25(OH)D can be affected by genetic variation of the major transporter, the vitamin D-binding protein[85–87]. This is seen as variations in vitamin D-binding protein concentration[86,88] as well as some vitamin D-binding protein phenotypes potentially having stronger binding abilities than others[89]. Serum 25(OH)D can also differ due to genetic variation in its key activation enzyme, CYP27B1[90], that converts 25(OH)D to the active form. Such genetic variant information was not collected in the VHM survey so is a potential confounding factor. Future studies in this area could include this information to provide a more complete picture.

A small proportion of our sample was from South Asia (1·6 %, *n* 56), an ethnic group associated with high rates of betel nut chewing. Chewing betel nut could increase the risk of developing T2DM[91] and animal studies have indicated that betel nut ingestion in male parents may contribute to inheritable glucose intolerance in their offspring[92]. Such data are not available for Australia and were not collected as part of the VHM survey. However, exclusion of these cases (*n* 56) did not change the direction or magnitude of our results. We therefore anticipate minimal confounding from such a potential habit in our South Asian participants.

Study strengths

We have used a large, representative, population-based sample of one Australian state that covered an age range 18–75 years. The dietary data were collected through a multiple-pass 24 h dietary recall which is the current standard and all blood analysis was conducted centrally by one laboratory based on standard methodology. Our analysis has considered and adjusted for many socio-demographic and nutrient confounders, with further adjustment for energy intake. We acknowledge that this field of research would benefit from the confirmation of a causal role for Ca and vitamin D in MetS. While randomized controlled trials provide Level 1 evidence, they are not necessarily the mainstay of the evidence base for public health nutrition and in deciding nutrition priorities for better health[93,94].

Conclusions

The present study demonstrates that high serum 25(OH)D concentration was associated with significant reductions in the odds of MetS. We raise the possibility that the benefit of Ca is restricted to low and medium serum 25(OH)D concentrations, and this may represent a threshold to the interplay between Ca and 25(OH)D on functional outcomes. Overall, these population-based results contribute to the evidence in favour of a role for vitamin D and Ca in modulation of MetS risk.

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Public Health Nutrition

Appendix D Vitamin D status is inversely associated with markers of risk for type 2 diabetes: a population based study in Victoria, Australia

PLOS ONE

RESEARCH ARTICLE

Vitamin D status is inversely associated with markers of risk for type 2 diabetes: A population based study in Victoria, Australia

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Abstract

A growing body of evidence suggests a protective role of vitamin D on the risk of type 2 diabetes mellitus (T2DM). We investigated this relationship in a population sample from one Australian state. The data of 3,393 Australian adults aged 18–75 years who participated in the 2009–2010 Victorian Health Monitor survey was analyzed. Socio-demographic information, biomedical variables, and dietary intakes were collected and fasting blood samples were analyzed for 25, hydroxycholecalciferol (25OHD), HbA1c, fasting plasma glucose (FPG), and lipid profiles. Logistic regression analyses were used to evaluate the association between tertiles of serum 25OHD and categories of FPG (<5.6 mmol/L vs. 5.6–6.9 mmol/L), and HbA1c (<5.7% vs. 5.7–6.4%). After adjusting for social, dietary, biomedical and metabolic syndrome (MetS) components (waist circumference, HDL cholesterol, triglycerides, and blood pressure), every 10 nmol/L increment in serum 25OHD significantly reduced the adjusted odds ratio (AOR) of a higher FPG [AOR 0.91, (0.86, 0.97); p = 0.002] and a higher HbA1c [AOR 0.94, (0.90, 0.98); p = 0.009]. Analysis by tertiles of 25OHD indicated that after adjustment for socio-demographic and dietary variables, those with high 25OHD (65–204 nmol/L) had reduced odds of a higher FPG [AOR 0.60, (0.43, 0.83); p = 0.008] as well as higher HbA1c [AOR 0.67, (0.53, 0.85); p = 0.005] compared to the lowest 25OHD (10–44 nmol/L) tertile. On final adjustment for other components of MetS, those in the highest tertile of 25OHD had significantly reduced odds of higher FPG [AOR 0.61, (0.44, 0.84); p = 0.011] and of higher HbA1c [AOR 0.74, (0.58, 0.93); p = 0.041] vs. low 25OHD tertile. Overall, the data support a direct, protective effect of higher 25OHD on FPG and HbA1c; two criteria for assessment of risk of T2DM.

Introduction

Vitamin D status, as judged from circulating concentrations of 25, hydroxycholecalciferol (25OHD), is a worldwide concern. In many countries across all continents, approximately

health-systems/health-status-of-victorians/survey-data-and-reports.

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50% of those populations have an inadequate 25OHD status (<50 nmol/L) [1]. Countries like India and China have some of the highest rates of vitamin D deficiency (<25 nmol/L) [1]. The 25OHD status of Australians is also surprisingly low for a country blessed with abundant sunshine. Current estimates indicate that ~31% of the population have inadequate 25OHD levels [2], with a higher prevalence in older Australians [3]. The prevalence of type 2 diabetes mellitus (T2DM) has also risen tremendously in the last 10 years, with projections that countries like India and China will have the highest numbers by 2030 at 79.4 and 42.3 million respectively [4].

There is an ongoing interest in the extra-skeletal effects of vitamin D including its potential to blunt the risk of developing T2DM. Positive outcomes would present a tangible public health solution, if causality is accepted. Such a relationship was first suggested in 1967 by Milner and Hales, who found that insulin secretion in rabbits was dependent on calcium and magnesium, which are tightly regulated by vitamin D [5]. Accumulating evidence has indicated that higher 25OHD status may have several anti-diabetic effects, including improvement in insulin sensitivity, stabilizing HbA1c levels [6], and improving beta cell function [7], whereas low 25OHD status may increase risk of T2DM [8]. Thus, in the current environment of increasing rates of T2DM [9], their close parallelism with insufficient levels of 25OHD deserves investigation in population based studies. There are several lifestyle factors that modulate the risk of T2DM, including dietary components and patterns [10, 11], physical activity, and smoking [12]. The risk of developing T2DM over 20 years appears to increase with the accumulation of metabolic syndrome (MetS) components. The risk of T2DM increased by: 11.9% in those with zero Mets components, 31.2% in those with three MetS components and 40.8% in those with four or five MetS components [13]. Though the presence of MetS components increases the risk of T2DM, glucose is the most strongly correlated factor in predicting the development of diabetes in the future [14]. In a study of more than 58,000 adults, as the number of components increased, so did the incidence of diabetes [14]. However, some gaps may exist with one study in Hispanic Americans finding that impaired glucose tolerance had a greater predictive power than the individual MetS components [15]. Thus, the presence of MetS is another major risk predictor of increased T2DM [16].

The aim of this study was to investigate the association of 25OHD, and the risk of T2DM. We [17] as well as others [18–21], have shown that higher 25OHD status significantly reduced the risk of MetS and its components. Hence it was essential to adjust for several lifestyle factors and components of MetS, other than glucose, in order to correctly identify any independent association between 25OHD and risk of T2DM. One other study [8] has also investigated the association between 25OHD and T2DM and adjusted for three out of the four MetS components. Thus our study appears to be one of the first to adjust for all MetS components. Impaired fasting plasma glucose (FPG) and HbA1c levels are now recommended as key determinants of early risk of T2DM [22]. While high FPG is an immediate indicator of poor glucose homeostasis, HbA1c is a better indicator of longer term control of blood glucose, and recommended cut-offs for both these biomarkers are used to diagnose T2DM [22]. The underlying hypothesis of the present investigation was that increases in 25OHD would reduce the odds ratio of a high FPG and a high HbA1c after adjustment for socio-demographic, dietary and biomedical confounders.

Materials and methods

Sample

The Victorian Health Monitor (VHM) survey was a state-wide cross-sectional population based study [23] conducted in Victoria, Australia. Victoria lies in the south-east of Australia, and has a

latitude of 37˚47'S and longitude of 144˚58'E. Data was collected between May 2009 and April 2010 including: physical information, dietary behaviour information and biomedical information. The physical and biomedical information of participants were collected by trained staff at four training sites. The VHM employed a stratified cluster sample selection method of Census Collection Districts within eight Department of Health regions in Victoria. Data were collected on 3,653 adults aged 18–75 years. From this sample, we excluded participants with: missing HbA1c and FPG data (n = 31), those with HbA1c $\geq$6.5% (n = 39) and FPG >7 mmol/L (n = 16) as they were classified as having T2DM as per the American Diabetes Association (ADA) cut-offs [22], those with T2DM (n = 140), those with type 1 diabetes (n = 9), and those on diabetic medications (n = 25). A total of 3,393 subjects were included in this analysis. Further details on physical, dietary, and biomedical data collection and analysis have been previously described [17, 23, 24].

Biomedical measurements

Participants attended a testing site after an overnight fast of at least 10 hours. Blood samples were collected by venepuncture, and were subsequently transported to a central laboratory in Melbourne, Australia. Bloods were analysed for: FPG, HbA1c, 25OHD, high density lipoprotein cholesterol (HDL-C), and triglycerides (TG). The components in the blood were measured as follows: FPG using the hexokinase method, HbA1c using immunosassay (Roche Integra chemistry analyser), 25OHD concentration were measured based on the DiaSorin Corporation Liaison® 25OHD total assay HDL-C using elimination/catalase method; and TG using GPO Trinder reagent set with serum blank. The blood pressure (BP) of participants was measured by survey staff using an automated BP monitor, which was calibrated regularly [23].

Physical measurements

The anthropometric measurements were made at the testing sites by trained staff, and included height, weight and waist circumference. Height was measured without shoes using a stadiometer. Weight was measured without shoes and light clothing, using a digital weighting scale. Waist circumference was measured using a steel measuring tape. Body mass index (BMI) was calculated from the weight and height measurements [23].

Dietary and physical activity measurements

Dietary information was collected by multiple-pass 24 h diet recall using computer assisted telephone interviews. Dietary recall interviews were conducted by dietitians from the Department of Nutrition and Dietetics at Monash University in Melbourne, Australia. The FoodWorks® nutrition software (FoodWorks® Interview) were used for conducting the dietary recalls. Based on the three dietary recalls, the mean intake for each nutrient was calculated and used in the analysis [23]. Further information on the assessment of dietary intake data can be found in our previous publications [17, 23]. Physical activity information was collected via interviews with the participant. The time spent in physical activity was calculated based on the sum of the time spent walking or performing moderate activity plus double the time spent in vigorous activity (to indicate its greater intensity) [23, 25].

FPG and HbA1c

Fasting plasma glucose and HbA1c were the two dependent variables. The ADA cut-offs were used to identify those who were at a risk of T2DM based on FPG and HbA1c levels[22]. A binary variable was used to categorize subjects as being at low or high risk for T2DM: FPG

<5.6 mmo/L (low risk, normal), vs. 5.6–6.9 mmol/L (high risk), and HbA1c <5.7% (low risk, normal) vs. 5.7–6.4% (high risk).

25OHD concentration

25OHD concentration was the primary independent variable. 25OHD concentration were categorized as tertiles: low 25OHD (median 33 nmol/L; range 10–44 nmol/l), medium 25OHD (median 54 nmol/L; range 45–65 nmol/L) and high 25OHD (median 77 nmol/L; range 65–204 nmol/L).

Statistical analysis

Socio-demographic factors. In our analysis we considered a number of confounders, based on our [26] and others experience in the area [12, 27]. We adjusted for the following socio-demographic factors: age, gender, county of birth, Index of Relative Socio-economic Disadvantage (IRSED), physical activity, smoking status, and season. Age were entered as continuous variables. Country of birth was categorized as those born in Australia or overseas. The socio-economic indicator used was the IRSED, which is an index based on the social and economic conditions of individuals within an area [28]. Subjects were categorized into IRSED quartiles: quartile 1 (most disadvantaged), quartile 2 (disadvantaged), quartile 3 (less disadvantaged), and quartile 4 (least disadvantaged). Physical activity levels were classified into three categories: sufficient activity (≥150 minutes/week), insufficient activity (1–149 minutes/week), and inactive (0 minutes/week). There is a known seasonal variation to FPG and HbA1c, so we had to adjust for season [29, 30]. Season of biomedical assessment refers to the season of the year that the participant attended the testing site, and had their bloods collected for assessment of 25OHD status. Season of biomedical assessment were grouped as summer, autumn, winter, and spring. Smoking status were categorized into three categories: current smoker, ex-smoker, and non-smoker.

Dietary factors. Based on previous research [31–33] dietary factors included in the analyses were: dietary fiber, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake and under/over reporting of energy intake. Self-reported energy intake may result in the under or over reporting of true energy intake (EI), and this may confound the estimation of any diet and disease related outcomes [34]. We predicted basal metabolic rate (BMR) using the Henry/Oxford equations based on a range of ages (18–30, 30–60, 60–70, and 70 and above years), gender and body weight [35]. Rather than use the Goldberg cut-offs to identify under-reporters and over-reporters [36], we calculated the ratio of energy intake to BMR (EI:BMR) and treated it as a confounder. Dietary fiber, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake, and under/over reporting of energy intake were all entered as continuous variables.

Biomedical factors. Biomedical factors included in the analyses were: MetS components including waist circumference, high density lipoprotein (HDL) cholesterol, triglycerides (TG), BP, as well as body mass index (BMI), and haemoglobin levels (HbA1c model only). Haemoglobin levels were adjusted for in the HbA1c model only, as recent findings have indicated that haemoglobin levels may increase HbA1c levels [37, 38]. BMI and haemoglobin levels were entered as a continuous variable. Those with MetS tend to be at higher risk of developing T2DM, thus we adjusted for MetS components in the HbA1c and FPG model [39]. MetS components were each classified as binary variables, as defined by the joint interim statement [40]. Waist circumference were: normal waist circumference (<94cm for males or if Aboriginal or Torres Strait Islander (ATSI), Asian or South American <90cm; <80cm for females), or elevated waist circumference (≥ 94cm for males or if ATSI, Asian or South American ≥90cm;

≥80cm for females). HDL cholesterol were: normal HDL (≥1.0 mmol/L for males; ≥1.3 mmol/L for females), or low HDL (<1,0 mmol/l for males; <1.3 mmol/L for females). TG were: normal TG (<1.7 mmol/l), or hypertriglyceridaemia (≥1.7 mmol/l). BP were: normal BP (<130/85 mmHg and no anti-hypertensive medications), or high BP (≥130/85 mmHG or on anti-hypertensive medications) [40].

Statistical analysis. The statistical analysis was conducted in the following two stages:

Step 1: Descriptive statistics for HbA1c and FPG were obtained and normality was assessed for variables of interest (natural logarithm transformation was applied if variable was skewed). Differences between groups were then examined by Independent samples *t* test and χ^2 test.

Step 2: Multiple logistic regression analyses were employed to obtain adjusted odds ratio (AOR) and 95% CI for the associations between serum 25OHD and having higher HbA1c and FPG, respectively. Three categories of variables were used in the regression models including: *socio-demographic variables* (age, sex, country of birth, IRSED, physical activity, smoking status, and season), *dietary factors* (dietary fiber, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake, and under/over reporting of energy intake) and *biomedical variables* (MetS components: waist circumference (ethnic specific cut-offs), HDL cholesterol (normal/low), TG (normal/high), BP (normal/high), BMI and haemoglobin levels for the HbA1c model only). All these variables have a known association with FPG and HbA1c and have been previously reported in the literature.

The primary research variable of interest, serum 25OHD, was entered into the multiple regression model as 1) a continuous variable (10 nmol/L increments) and also as 2) a categorical variable based on 25OHD tertiles. For both continuous and categorical 25OHD, the multiple regression model was initially adjusted for the socio-demographic variables in Model 1; secondly both socio-demographic variables, dietary factors and haemoglobin levels (HbA1c model only) in Model 2; and finally all socio-demographic, dietary factors and biomedical variables altogether in Model 3. The IBM SPSS Statistics for Windows, Version 21.0, was used for the statistical analyses. The VHM survey employed the use of the multistage stratified cluster-sampling procedure. In order to adjust for the unequal selection probability due to this sampling method, complex samples analysis was used. Complex samples approach takes into account the complex survey sampling and selection probability used in the VHM survey. Variables (strata variable, weighting variable and clustering variable) describing the survey design in terms of stratification, clustering and multistage sampling were entered into the SPSS complex samples approach for generating sampling weights in estimation and standard errors. A two-tailed p value of less than 0.05 was accepted as statistical significance.

Results

An overview of socio-demographic, dietary and clinical characteristics of subjects based on FPG and HbA1c levels are shown in Table 1 (please refer to S1 Table for the full socio-demographic, dietary and clinical characteristics of subjects). The prevalence of those with normal FPG (<5.6 mmol/L) was 84% and for high FPG (5.6–6.9 mmol/L) was 16%. 61% of the population had normal HbA1c (<5.6%), while 39% had high HbA1c (5.7–6.4%).

Association between serum 25OHD and FPG

When the serum 25OHD was entered as a continuous covariate to the multiple regression model, for every increment in serum 25OHD of 10 nmol/L, the odds of having higher FPG reduced by 9% (AOR 0.91, (0.86, 0.97); p = 0.002) after adjusting for socio-demographic variables, dietary factors, and biomedical variables in Model 3. For all models when serum 25OHD was entered as a categorical factor, compared with those people in the low 25OHD

Table 1. Socio-demographic and clinical characteristics of participants by FPG and HbA1c.

	FPG (<5.6 mmol/L) n = 2866 (84%)	FPG (5.6–6.9 mmol/L) n = 527 (16%)	P value	HbA1c (<5.7%) n = 2068 (61%)	HbA1c (5.7–6.4%) n = 1325 (39%)	P value
	Mean (SE) *or* N (SE) %	Mean (SE) *or* N (SE) %		Mean (SE) *or* N (SE) %	Mean (SE) *or* N (SE) %	
Age (y)	42 (0.8)	52 (1.4)	<0.001	40 (0.9)	52 (0.9)	<0.001
BMI (kg/m^2)	26.6 (0.2)	29.2 (0.4)	<0.001	26.2 (0.2)	28.8 (0.2)	<0.001
Gender			<0.001			0.494
Males	1285 (1.5) 81%	299 (1.5) 19%		1112 (2.5) 70%	472 (2.5) 30%	
Females	1663 (1.0) 91%	162 (1.0) 9%		1257 (2.4) 69%	577 (2.4) 31%	
Country of birth			0.031			<0.001
Born in Australia	2264 (0.7) 88%	320 (0.7) 12%		1868 (2.1) 72%	716 (2.1) 28%	
Born overseas	674 (1.9) 83%	134 (1.9) 17%		505 (3.6) 62%	303 (3.6) 38%	
IRSED			0.020			0.216
Most disadvantaged	705 (1.7) 83%	142 (1.7) 17%		545 (5.1) 64%	302 (5.1) 36%	
Disadvantaged	732 (1.0) 87%	108 (1.0) 13%		570 (3.8) 68%	270 (3.8) 32%	
Less disadvantaged	765 (1.3) 89%	98 (1.3) 11%		639 (4.1) 74%	224 (4.1) 26%	
Least disadvantaged	737 (1.2) 87%	106 (1.2) 13%		631 (3.3) 75%	212 (3.3) 25%	
Smoking status			<0.001			0.002
Current smoker	441 (2.2) 84%	86 (2.2) 16%		366 (3.7) 69%	161 (3.7) 31%	
Ex-smoker	733 (2.0) 80%	179 (2.0) 20%		575 (3.2) 63%	337 (3.2) 37%	
Non-smoker	1756 (0.9) 90%	195 (0.9) 10%		1418 (2.1) 73%	533 (2.1) 27%	
25OHD concentration						
Serum 25OHD (nmol/L)	56.7 (2.0)	52.1 (2.5)	0.081	57.2 (2.2)	53.6 (1.9)	0.208
25OHD tertiles			0.045			0.135
Low 25OHD (33 nmol/L)	933 (1.5) 84%	180 (1.5) 16%		745 (2.9) 67%	359 (2.9) 33%	
Medium 25OHD (54 nmol/L)	992 (1.3) 85%	168 (1.3) 15%		798 (3.5) 69%	32 (3.5) 31%	
High 25OHD (77 nmol/L)	1013 (1.5) 90%	116 (1.5) 10%		829 (2.4) 73%	300 (2.4) 27%	
Dietary variable						
Energy (kJ/d)	9687.4 (116.5)	9784.9 (164.9)	0.653	9904.4 (147.8)	9236.4 (145.6)	<0.001
Biomedical factors						
Waist circumference (cm)	88.0 (0.7)	96.9 (1.1)	<0.001	86.9 (0.7)	94.7 (0.9)	<0.001
Triglycerides (mmol/L)	1.2 (0.03)	1.5 (0.04)	<0.001	1.1 (0.03)	1.5 (0.04)	<0.001
HDL (mmol/L)	1.5 (0.02)	1.4 (0.03)	<0.001	1.5 (0.02)	1.4 (0.02)	<0.001
Systolic blood pressure (mmHg)	123 (0.6)	133 (1.1)	<0.001	123 (0.7)	128 (0.6)	<0.001
Diastolic blood pressure (mmHg)	73 (0.5)	77 (0.7)	<0.001	72 (0.5)	76 (0.5)	<0.001
Haemoglobin levels (g/L)	142.9 (0.4)	148.2 (1.1)	<0.001	144.2 (0.4)	142.4 (0.6)	<0.001

Data are presented as mean estimate (weighted) (%) for categorical variables, and mean estimate (weighted) and (SE) for normal continuous variables. Difference in the continuous and categorical variables between groups were assessed by independent samples t-test (natural logarithm transformation was used if the variable was not normal) and Chi-square test (association between FPG or HbA1c and categorical variables, with an emphasis on which category were more likely to have high FPG, or high HbA1c), respectively.

Legend: d, day; SE, standard error; min, minutes; wk, week.

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tertile, those with the high 25OHD tertile had a significantly reduced risk of higher FPG. More specifically, after adjustment for both socio-demographic, dietary factors and BMI in Model 2 the odds of having higher FPG reduced by 40% for those in high 25OHD tertile (AOR 0.60, (0.43, 0.83); p = 0.008) vs. low 25OHD tertile. After further adjustment for MetS components

Table 2. The association of serum 25OHD and FPG: Crude and adjusted odds ratio and their 95% CI based on logistic regression.

	Model 1		Model 2		Model 3	
	Crude OR	**95% CI**	**AOR**	**95% CI**	**AOR**	**95% CI**
25OHD continuous (10 nmol/L)	0.93	0.87, 1.01	0.91	0.86, 0.97	0.91	0.86, 0.97
P value	0.054		0.003		0.002	
	Model 1		**Model 2**		**Model 3**	
25OHD tertiles	**Crude OR**	**95% CI**	**AOR**	**95% CI**	**AOR**	**95% CI**
Low 25OHD (33 nmol/L) †	1.0		1.0		1.0	
Medium 25OHD (54 nmol/L) †	0.89	0.60, 1.31	0.87	0.57, 1.32	0.87	0.59, 1.29
High 25OHD (77 nmol/L) †	0.66*	0.46, 0.94	0.60*	0.43, 0.83	0.61*	0.44, 0.84
P value for trend	0.076		0.008		0.011	

Model 1: age, sex, country of birth, IRSED, physical activity, smoking status, season, BMI.

Model 2: Model 1 plus dietary fiber, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake, under/over reporting of energy intake.

Model 3: Model 2 plus waist circumference, HDL cholesterol, TG, BP; all as categorical variables based on MetS cut-offs.

Legend: Crude OR, crude odds ratio; AOR, adjusted odds ratio; 1.0, lowest 25OHD served as the reference group.

Footnotes

*, significant in comparison to reference group at 5% significance level

†, median of the tertile group.

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in Model 3, the AOR appeared relatively stable with a 39% reduced odds of higher FPG in the high 25OHD vs. low 25OHD tertile (AOR 0.61, (0.44, 0.84); p = 0.011) (Table 2).

Association between serum 25OHD and HbA1c

In Model 3 after adjustment for socio-demographic, dietary, and biomedical variables every 10 nmol/L increment in 25OHD also significantly reduced the odds of higher HbA1c by 6% (AOR 0.94, (0.90, 0.98); p = 0.009). In Model 2, after adjustment for socio-demographic variables, dietary factors, BMI and haemoglobin levels, there was a significantly reduced odds of having higher HbA1c by 33% in those with high vs. low 25OHD tertile. After further adjustment for MetS components in Model 3, a 26% reduced odds of higher HbA1c (AOR 0.74, (0.58, 0.93); p = 0.041) were found in the high 25OHD tertile group, compared to the low 25OHD tertile group (Table 3).

Discussion

The results from this population based study of adults from Victoria, Australia indicate that higher 25OHD levels were significantly related to a reduced risk of higher FPG and HbA1c levels. This significant inverse association persisted after the adjustment for a number of socio-demographic, dietary, and biomedical variables and MetS components. We found that those in the high 25OHD tertile had a 39% reduced risk of higher FPG and 26% reduced risk of higher HbA1c, when compared to the low 25OHD tertile. This was independent of MetS components, and haemoglobin levels in the HbA1c model, which have been found to confound the association between 25OHD and T2DM. Two recent meta-analyses [41–42] found no significant effect of vitamin D supplementation on IR [41], FPG [41, 42] or HbA1c [42]. Though, another found a beneficial effect of vitamin D supplementation on FPG and HbA1c, despite no effect on IR [43]. Our recent review summarised that although there were inverse associations between 25OHD and IR, the systematic reviews and meta-analysis in that review did not favour a casual role [44]. The present findings are in line with previous observational studies that found an inverse association between 25OHD status and high FPG levels [20, 45] and

Table 3. The association of serum 25OHD and HbA1c: Crude and adjusted odds ratio and their 95% CI based on logistic regression.

	Model 1		Model 2		Model 3	
	Crude OR	**95% CI**	**AOR**	**95% CI**	**AOR**	**95% CI**
25OHD continuous (10 nmol/L)	0.93	0.88, 0.97	0.93	0.88, 0.97	0.94	0.90, 0.98
P value	0.002		0.002		0.009	
	Model 1		**Model 2**		**Model 3**	
25OHD tertiles	**Crude OR**	**95% CI**	**AOR**	**95% CI**	**AOR**	**95% CI**
Low 25OHD (33 nmol/L) †	1.0		1.0		1.0	
Medium 25OHD (54 nmol/L) †	0.78	0.56, 1.09	0.79	0.56, 1.11	0.83	0.58, 1.17
High 25OHD (77 nmol/L) †	0.68*	0.54, 0.86	0.67*	0.53, 0.85	0.74*	0.58, 0.93
P value for trend	0.007		0.005		0.041	

Model 1: age, sex, country of birth, IRSED, physical activity, smoking status, season, BMI.

Model 2: dietary fiber, magnesium, alcohol, calcium, zinc, carbohydrate intake, energy intake, under/over reporting of energy intake, haemoglobin levels.

Model 3: Model 2 plus waist circumference, HDL cholesterol, TG, BP; all as categorical variables based on MetS cut-offs.

Legend: Crude OR, crude odds ratio; AOR, adjusted odds ratio; 1.0, lowest 25OHD served as the reference group.

Footnotes

*, significant in comparison to reference group at 5% significance level

†, median for the tertile group.

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high HbA1c levels [46–51]. However, these studies adjusted for fewer variables, with smaller sample sizes than our study and were not population based [45, 49–51]. The differences in findings between both interventional and observational based studies indicates a need for good quality randomized controlled trials.

A number of inter-related factors contribute to the pathogenesis of T2DM, including the presence of MetS components. One prospective study found that those with IR and high FPG (5.6–6.9 mmol/L) had double the risk of worsened cardio-metabolic profile after nine years [52]. Obesity, dyslipidemia and hypertension are MetS components which are often found in those with pre-diabetes [53]. We adjusted for these variables in our analysis and found that the association between 25OHD and risk of T2DM still existed, irrespective of MetS. Other studies investigating 25OHD and risk of T2DM have adjusted for either none [47–49], or one MetS components [46, 54, 55], with one study accounting for all of the MetS components [8].

Potential mechanisms

The beneficial effect of 25OHD in reducing risk of T2DM is likely due to its effect on insulin action. The expression of the vitamin D receptor (VDR) in pancreatic beta cells indicates the importance of vitamin D in beta cell function [56, 57] and insulin secretion [5]. During times of vitamin D deficiency, beta cell function is blunted and insulin secretion is diminished [58]. Vitamin D may also indirectly influence insulin action via a calcium mediated effect. Vitamin D tightly regulates calcium homeostasis, whereby intracellular calcium levels are required to ensure effective action of insulin within different tissues [56]. Vitamin D is also involved in the regulation of the renin angiotensin system [59], endothelial vasodilation and lipid levels [60] which are mechanisms relating to the MetS components. T2DM and MetS are inter-twined, wherein IR appears to be a key player in the development of both conditions [61, 62]. Though there are commonalties in the mechanisms underlying both conditions, our study found that even on adjustment for MetS components, the association between the high 25OHD tertile and lower odds of higher FPG and HbA1c levels persisted. This may potentially suggest that vitamin D has a beneficial role in T2DM, independent of MetS. However, the cross-sectional nature of this study does not provide further insight into this observation.

Low grade chronic inflammation is a hallmark of many chronic diseases and may precede T2DM [63] possibly via initiation of IR [64]. Vitamin D has a role in immunity, and cellular studies show consistent reduction of inflammatory markers following cholecalciferol supplementation [65]. Adequate circulating 25OHD levels are required to obtain optimal anti-inflammatory responses in the body [66], especially in those tissues like immune cells, where the enzymes for conversion of 25OHD to 1,25OH_2D are present. However, the optimum 25OHD levels for modulating inflammation responses are yet to be determined [67]. There is plausible but not confirmatory evidence to suggest the value is around 75–80 nmol/L [68, 69]; a point where maximum suppression of parathyroid hormone is also expected [70]. In support, a recent review [71], and emerging randomized controlled trials [72, 73] found beneficial effects of vitamin D supplementation on IR and fat loss in those individuals who reached this value over the period of the trial [72, 73]. In the present study those in the highest 25OHD tertile had a median of 77 nmol/L, where reduced risk of higher FPG and HbA1c was observed.

Limitations

The cross-sectional design does not afford causality of association, though we have controlled for several known confounders. The VHM did not collect information on supplement use, so we cannot separate the potential effects of vitamin D supplement and increased sun exposure on the higher 25OHD levels. Family history of T2DM may increase the risk of development of the disease [74], but unfortunately such information was not collected as part of the survey thus is a potential confounder. Approximately >75% of our sample were born in Australia, so potential effects of country of birth/ethnicity could not be determined.

Studies have indicated that 25OHD may vary due to the genetic variation of three polymorphisms in the vitamin D genes, including the vitamin D binding protein, VDR and the 25OHD activating enzyme [75, 76]. The concentration of the vitamin D binding protein may vary between individuals [77, 78] as well as fluctuations in its binding affinity [79]. There may also be a genetic association between VDR polymorphisms and T2DM. Recent evidence has found that the polymorphism of certain VDRs may increase susceptibility to T2DM [80] and certain MetS components [81]. Lastly, the conversion of 25OHD to its active form requires CYP27B1, an enzyme that may be affected by genetic variation [82]. These genetic variants were not studied as part of the VHM survey but certainly provide avenues for novel insights into the associations described here.

Strengths

This study used a representative sample of adult Australians from one state of the country. We adjusted for a wide range of socio-demographic, biomedical and dietary factors. Our study appears to be one of the few [8] which has accounted for all MetS components when investigating associations between 25OHD and T2DM. We also adjusted for the possible misreporting of energy intakes [35]. That being said 24 h recalls in this study were obtained from a five-pass method which is considered the gold standard for dietary information. We also adjusted the HbA1c analysis for haemoglobin concentrations, since concomitant anaemia may be associated with inaccurate HbA1c levels [37, 38]. Other studies in the area have not adjusted for this factor [8, 46–49, 54, 55].

Conclusions

Higher 25OHD status was associated with lower prevalent FPG as well as lower HbA1c concentrations after accounting for socio-demographic, lifestyle variables and MetS components. Such outcomes could suggest a direct role for the vitamin in the prevention of T2DM.

Supporting information

S1 Table. Socio-demographic and clinical characteristics of participants by FPG and HbA1c. Data are presented as mean estimate (weighted) (%) for categorical variables, and mean estimate (weighted) and (SE) for continuous variables. Difference in the continuous and categorical variables between groups were assessed by independent samples t-test and Chi-square test, respectively. Legend: d, day; SE, standard error; min, minutes; wk, week.
(DOCX)

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Visualization: PKP LSP YZ ZA.

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Appendix F Permission from authors: Calcium and vitamin D in obesity and related chronic disease

I, Poonam Kaur Pannu, contributed (conducted literature search and co-wrote manuscript) to the paper/publication entitled:

Pannu, P. K., Calton, E. K., & Soares, M. J. (2016). Calcium and vitamin D in obesity and related chronic disease. In J. Henry (Ed.), *Advances in Food and Nutrition Research, volume 77*. London: Academic Press.

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Co-author 1: Emily K Calton

Co-author 2: Mario J Soares

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Appendix H Permission from authors: Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects: a systematic review and metaregression analysis

I, Poonam Kaur Pannu, contributed (conducted literature search, assembled tables, extracted the data and co-wrote manuscript) to the paper/publication entitled:

Pannu, P. K., Zhao, Y., & Soares, M. J. (2016). Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects: a systematic review and metaregression analysis. *Nutrition Research, 36*(3), 201-213. doi:10.1016/j.nutres.2015.11.013

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Co-author 1: Yun Zhao

Co-author 2: Mario J Soares

Appendix I Permission from publisher: The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor survey

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Wednesday, 27 July 2016

Dear Poonam K. Pannu,

Poonam K Pannu, Yun Zhao, Mario J Soares, Leonard S Piers & Zahid Ansari (2016) 'The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor survey, *Public Health Nutrition*, Cambridge University Press, Research Paper, pp. 1-12

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Pannu, P. K., Zhao, Y., Soares, M. J., Piers, L. S., & Ansari, Z. (2016). The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor survey. *Public Health Nutrition*, 1-12. doi:10.1017/S1368980016001609

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Co-author 1: Yun Zhao

Co-author 2: Mario J Soares

Co-author 3: Leonard S Piers

Co-author 4: Zahid Ansari

Appendix K Permission from authors: The influence of calcium and vitamin D on components of the metabolic syndrome: an update of current evidence

I, Poonam Kaur Pannu, contributed (conducted literature search and co-wrote manuscript) to an unpublished paper entitled:

Pannu P. K., Soares, M. J., Pathak, K., & Calton, E. K. (Submitted 16/03/2017). The influence of calcium and vitamin D on components of the metabolic syndrome: an update of current evidence. *Cardiology Research and Practice*, Manuscript ID: 2185648.

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Co-author 2: Emily K Calton

Co-author 3: Kaveri Pathak

Appendix L Permission from authors: The association of vitamin D status and dietary calcium intake with individual components of the metabolic syndrome: a population based study in Victoria, Australia

I, Poonam Kaur Pannu, contributed (planned and conducted analysis, and co-wrote manuscript) to the paper/publication entitled:

Pannu, P. K., Soares, M. J., Piers, L. S., Zhao, Y., & Ansari, Z. (Submitted 23/05/2017). The association of vitamin D status and dietary calcium intake with individual componenets of the metabolic syndrome: a population based study in Victoria, Australia. *Cardiovascular Endocrinology*. Manuscript ID: CAEN-D-17-00009.

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Pannu, P. K., Piers, L. S., Soares, M. J., Zhao, Y., & Ansari, Z. (2017). Vitamin D status is inversley associated with markers of risk for type 2 diabetes: a population based study in Victoria, Australia. *PloS ONE, 12*(2), e0178825. doi: 10.1371/journal.pone.0178825.

Poonam K Pannu

Co-author 1: Leonard S Piers

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Appendix O Papers published during PhD

1. Calton, E. K., James, A. P., **Pannu, P. K.**, & Soares, M. J. (2014). Certain dietary patterns are beneficial for the metabolic syndrome: reviewing the evidence. *Nutrition Research, 34*(7), 559-568. doi:10.1016/j.nutres.2014.06.012

- Number of citations 2014-2017: 49.

2. **Pannu, P. K.**, Zhao, Y., & Soares, M. J. (2016). Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects: a systematic review and metaregression analysis. *Nutrition Research, 36*(3), 201-213. doi:10.1016/j.nutres.2015.11.013

- Number of citations 2016-2017: 15.

3. **Pannu, P. K.**, Zhao, Y., Soares, M. J., Piers, L. S., & Ansari, Z. (2016). The associations of vitamin D status and dietary calcium with the metabolic syndrome: an analysis of the Victorian Health Monitor survey. *Public Health Nutrition,* 1-12. doi:10.1017/S1368980016001609

- Number of citations 2016-2017: 3.

4. **Pannu, P. K.**, Calton, E. K., & Soares, M. J. (2016). Calcium and vitamin D in obesity and related chronic disease. In J. Henry (Ed.), *Advances in Food and Nutrition Research,* (Vol. 77). London: Academic Press.

- Number of citations 2016-2017: 9.

5. Soares, M. J., **Pannu, P. K.**, Calton, E. K., Reid, C. M., & Hills, A. P. (2017). Vitamin D status and calcium intake in systemic inflammation, insulin resistance and the metabolic syndrome: an update on current evidence. *Trends in Food Science and Technology, 62,* 79-90. doi:10.1016/j.tifs.2017.01.009

- Number of citations 2017-current: 1.

6. **Pannu, P. K.**, Piers, L. S., Soares, M. J., Zhao, Y., & Ansari, Z. (2017). Vitamin D status is inversely associated with markers of risk for type 2 diabetes: a population based study in Victoria, Australia. *PloS ONE, 12*(2), e0178825. doi: 10.1371/journal.pone.0178825
7. **Pannu, P. K.**, Soares, M. J., Piers, L. S., Zhao, Y., & Ansari, Z. (2017). The association of vitamin D status and dietary calcium intake with individual components of the metabolic syndrome: a population based study in Victoria, Australia. Submitted to: *Cardiovascular Endocrinology*, Manuscript ID: CAEN-D-17-00009.

Appendix P Chapter 3 tables

Table P.1 Interactive model of 25OHD tertile and MFS in those born overseas.

	Low 25OHD (33 nmol/L)		Medium 25OHD (54 nmol/L)		High 25OHD (77 nmol/L)		P value
	OR	95% CI	OR	95% CI	OR	95% CI	
Modified Fitzpatrick Scale[a]							0.003
Dark brown or black skin colour	Ref.						
Brown skin colour			2.11	0.96, 4.66	2.83	0.88, 9.13	
Light brown skin colour			2.94*	1.09, 7.90	3.67*	1.10, 12.23	
Fair skin colour			5.84*	2.37, 14.41	8.66*	2.82, 26.62	

Model was adjusted for BMI, physical activity, smoking, sitting time, MFS, season, living area, dietary calcium intake.

Footnotes: Ref., reference category; *, significant in comparison to reference group at 5% significance level; [a], MFS score 4-5=dark brown or black skin colour; MFS score 6-7=brown skin colour, MFS score 8-9=light brown skin colour, MFS score 10-12=fair skin colour.

Table P.2 Interactive model of 25OHD tertile and MFS in those born in Australia.

	Low 25OHD (33 nmol/L)		Medium 25OHD (54 nmol/L)		High 25OHD (77 nmol/L)		P value
	OR	95% CI	OR	95% CI	OR	95% CI	
Modified Fitzpatrick Scale[a]							0.296
Dark brown or black skin colour	Ref.						
Brown skin colour			1.18	0.47, 7.15	2.62	0.77, 8.94	

	Low 25OHD (33 nmol/L)	Medium 25OHD (54 nmol/L)		High 25OHD (77 nmol/L)		P value
Light brown or olive skin colour		2.68	0.63, 11.42	4.19*	1.17, 14.98	
Fair skin colour		2.07	0.53, 8.05	3.67	0.92, 12.30	

Figure 3.5 Model was adjusted for BMI, physical activity, smoking, sitting time, MFS, season, living area, dietary calcium intake.

Footnotes: Ref., reference category; *, significant in comparison to reference group at 5% significance level; [a], MFS score 4-5=dark brown or black skin colour; MFS score 6-7=brown skin colour, MFS score 8-9=light brown skin colour, MFS score 10-12=fair skin colour.

Appendix Q Chapter 6 tables

Table Q.1 Selected demographic characteristics for elevated WC (yes/no).

	Elevated WC		
	No (normal)	Yes	P value
	N (SE) % *or* Mean (SE)	N (SE) % *or* Mean (SE)	
Gender			0.001
Males	799 (2.9) 51%	777 (2.9) 49%	
Females	761 (2.2) 42%	1050 (2.2) 58%	
Country of birth			0.524
Born in Australia	1179 (2.6) 46%	1401 (2.6) 54%	
Born overseas	386 (3.1) 48%	421 (3.1) 52%	
Age (y)	39 (0.8)	46 (1.2)	<0.001
Body weight (kg)	68.6 (0.6)	87.1 (0.6)	<0.001
Serum 25OHD (nmol/L)	58.4 (2.4)	54.2 (2.0)	0.084
Dietary calcium intake (mg)	940.8 (18.0)	887.6 (9.3)	0.005
Total energy intake (kJ)	10248.3 (143.5)	9262.2 (88.4)	<0.001

Table Q.2 Selected demographic characteristics for reduced HDL-C (yes/no).

	Reduced HDL-C		
	No (normal)	Yes	P value
	N (SE) % *or* Mean (SE)	N (SE) % *or* Mean (SE)	
Gender			0.004
Males	1397 (1.4) 89%	178 (1.4) 11%	
Females	1489 (2.0) 82%	323 (2.0) 18%	

Reduced HDL-C			
Country of birth			0.863
Born in Australia	2206 (1.4) 85%	374 (1.4) 15%	
Born overseas	687 (2.1) 85%	120 (2.1) 15%	
Age (y)	43 (1.0)	43 (0.9)	0.524
Body weight (kg)	77.6 (0.6)	84.2 (1.3)	<0.001
Serum 25OHD (nmol/L)	56.7 (1.9)	52.6 (2.8)	0.041
Dietary calcium intake (mg)	928.4 (10.5)	817.9 (24.4)	<0.001
Total energy intake (kJ)	9896.8 (118.4)	8666.1 (240.6)	<0.001

Table Q.3 Selected demographic characteristics for elevated TG (yes/no).

Elevated TG			
	No (normal)	Yes	P value
	N (SE) % *or* Mean (SE)	N (SE) % *or* Mean (SE)	
Gender			<0.001
Males	1157 (2.1) 73%	419 (2.1) 27%	
Females	1554 (1.2) 86%	257 (1.2) 14%	
Country of birth			0.455
Born in Australia	2064 (1.2) 80%	516 (1.2) 20%	
Born overseas	637 (1.3) 79%	170 (1.7) 21%	
Age (y)	42 (0.9)	47 (1.3)	<0.001
Body weight (kg)	76.3 (0.6)	87.1 (1.1)	<0.001
Serum 25OHD (nmol/L)	58.4 (2.1)	47.3 (2.2)	<0.001
Dietary calcium intake (mg)	915.4 (11.6)	899.5 (23.9)	0.178
Total energy intake (kJ)	9657.9 (137.5)	9943.1 (208.9)	0.044

Table Q.4 Selected demographic characteristics for elevated SBP (yes/no).

	Elevated SBP		
	No (normal)	Yes	P value
	N (SE) % *or* Mean (SE)	N (SE) % *or* Mean (SE)	
Gender			<0.001
Males	821 (2.5) 52%	755 (2.5) 48%	
Females	1358 (1.7) 75%	453 (1.7) 25%	
Country of birth			0.897
Born in Australia	1646 (2.2) 64%	934 (2.2) 36%	
Born overseas	512 (2.5) 63%	295 (2.5) 37%	
Age (y)	39 (0.8)	50 (1.1)	<0.001
Body weight (kg)	74.9 (0.7)	84.9 (0.6)	<0.001
Serum 25OHD (nmol/L)	56.7 (2.2)	55.1 (1.8)	0.463
Dietary calcium intake (mg)	920.9 (13.8)	897.1 (14.2)	0.093
Total energy intake (kJ)	9618.1 (135.7)	9885.0 (125.7)	0.056

Table Q.5 Selected demographic characteristics for elevated DBP (yes/no).

	Elevated DBP		
	No (normal)	Yes	P value
	N (SE) % *or* Mean (SE)	N (SE) % *or* Mean (SE)	
Gender			<0.001
Males	1176 (2.3) 75%	400 (2.3) 25%	
Females	1512 (1.3) 83%	299 (1.3) 17%	
Country of birth			0.069
Born in Australia	2061 (1.5) 80%	518 (1.5) 20%	
Born overseas	620 (1.9) 77%	187 (1.9) 23%	
Age (y)	40 (0.9)	54 (0.7)	<0.001
Body weight (kg)	75.9 (0.4)	88.5 (0.9)	<0.001

Elevated DBP			
Serum 25OHD (nmol/L)	56.9 (2.1)	53.0 (2.0)	0.066
Dietary calcium intake (mg)	927.4 (12.1)	855.1 (14.5)	<0.001
Total energy intake (kJ)	9805.1 (137.4)	9385.5 (122.8)	0.004

Table Q.6 Selected demographic characteristics for elevated FPG (yes/no).

Elevated FPG			
	No (normal) N (SE) % *or* Mean (SE)	Yes N (SE) % *or* Mean (SE)	P value
Gender			<0.001
Males	1278 (1.5) 81%	298 (1.5) 19%	
Females	1648 (1.0) 91%	163 (1.0) 9%	
Country of birth			0.050
Born in Australia	2247 (0.8) 87%	333 (0.8) 13%	
Born overseas	671 (2.0) 83%	136 (2.0) 17%	
Age (y)	42 (0.8)	52 (1.4)	<0.001
Body weight (kg)	77.2 (0.5)	86.6 (1.2)	<0.001
Serum 25OHD (nmol/L)	56.8 (2.0)	52.1 (2.4)	0.027
Dietary calcium intake (mg)	917.4 (11.9)	879.7 (21.8)	0.003
Total energy intake (kJ)	9697.2 (116.3)	9835.2 (158.4)	0.242

Table Q.7 Model 1: Unadjusted odds ratio of having MetS components by combination of 25OHD and dietary Ca intake.

	Low 25OHD (33 nmol/L)†		**Medium 25OHD (54 nmol/L)†**		**High 25OHD (77 nmol/L)†**		
	AOR	95% CI	AOR	95% CI	AOR	95% CI	P value
Low calcium (579 mg)†							
Elevated WC	1.0		1.08	0.68, 1.49	0.83	0.47, 1.45	0.051

	Low 25OHD (33 nmol/L)†		Medium 25OHD (54 nmol/L)†		High 25OHD (77 nmol/L)†		
Reduced HDL-C	1.0		0.42*	0.24, 0.75	0.43*	0.27, 0.70	0.001
Elevated TG	1.0		0.60*	0.41, 0.90	0.19*	0.13, 0.29	<0.001
Elevated SBP	1.0		1.29	0.98, 1.71	0.81	0.53, 1.25	0.133
Elevated DBP	1.0		1.02	0.70, 1.49	0.87	0.56, 1.36	<0.001
Elevated FPG	1.0		1.24	0.78, 1.97	0.54	0.30, 0.99	0.058
Medium calcium (858 mg)†							
Elevated WC	1.01	0.68, 1.49	0.88	0.55, 1.41	0.55	0.33, 0.94	0.051
Reduced HDL-C	0.39*	0.24, 0.63	0.47*	0.28, 0.78	0.38*	0.20, 0.72	0.001
Elevated TG	0.77	0.49, 1.20	0.61*	0.39, 0.93	0.34*	0.19, 0.60	<0.001
Elevated SBP	1.07	0.67, 1.71	0.95	0.66, 1.37	0.85	0.55, 1.31	0.133
Elevated DBP	0.95	0.60, 1.50	0.79	0.54, 1.16	0.44*	0.28, 0.69	<0.001
Elevated FPG	1.01	0.62, 1.63	0.71	0.43, 1.16	0.60	0.32, 1.11	0.058
High calcium (1233 mg)†							
Elevated WC	0.64	0.39, 1.04	0.91	0.49, 1.68	0.59	0.34, 1.00	0.051
Reduced HDL-C	0.27*	0.14, 0.52	0.25*	0.13, 0.48	0.30*	0.17, 0.52	0.001
Elevated TG	0.60	0.33, 1.08	0.65*	0.45, 0.94	0.31*	0.18, 0.54	<0.001
Elevated SBP	0.79	0.47, 1.33	0.91	0.64, 1.30	0.75	0.52, 1.08	0.133
Elevated DBP	0.66	0.43, 1.01	0.68	0.45, 1.04	0.51*	0.32, 0.80	<0.001
Elevated FPG	0.93	0.52, 1.65	0.64	0.38, 1.06	0.49	0.27, 0.88	0.058

Footnote: †Median 25OHD concentration and dietary calcium intake in each tertile; *significant as compared to the reference category.

Table Q.8 Model 2: Adjusted odds ratio of having MetS components by combination of tertiles of 25OHD and tertiles of dietary Ca intake.

	Low 25OHD (33 nmol/L)†		Medium 25OHD (54 nmol/L)†		High 25OHD (77 nmol/L)†		
	AOR	95% CI	AOR	95% CI	AOR	95% CI	P value
Low calcium (579 mg)†							
Elevated WC	1.0		0.89	0.04, 1.99	0.54	0.24, 1.21	0.417
Reduced HDL-C	1.0		0.39*	0.24, 0.64	0.40*	0.22, 0.72	0.034
Elevated TG	1.0		0.51*	0.34, 0.76	0.14*	0.09, 0.23	<0.001
Elevated SBP	1.0		1.18	0.81, 1.73	0.77	0.47, 1.28	0.130
Elevated DBP	1.0		0.89	0.48, 1.64	0.85	0.53, 1.39	0.009
Elevated FPG	1.0		1.26	0.73, 2.15	0.51	0.26, 1.03	0.057
Medium calcium (858 mg)†							
Elevated WC	0.92	0.56, 1.50	0.62	0.33, 1.14	0.65	0.32, 1.32	0.417
Reduced HDL-C	0.49*	0.30, 0.81	0.50*	0.27, 0.91	0.43*	0.22, 0.82	0.034
Elevated TG	0.76	0.42, 1.36	0.52*	0.33, 0.81	0.32*	0.19, 0.55	<0.001
Elevated SBP	0.94	0.55, 1.62	0.81	0.47, 1.39	1.02	0.60, 1.71	0.130
Elevated DBP	0.93	0.53, 1.63	0.74	0.42, 1.29	0.50*	0.32, 0.74	0.009
Elevated FPG	1.07	0.62, 1.85	0.68	0.37, 1.24	0.73	0.38, 1.39	0.057
High calcium (1233 mg)†							
Elevated WC	1.03	0.53, 1.98	0.69	0.30, 1.61	0.65	0.31, 1.43	0.417
Reduced HDL-C	0.36*	0.16, 0.79	0.28*	0.13, 0.61	0.38*	0.17, 0.85	0.034
Elevated TG	0.60	0.32, 1.12	0.56*	0.36, 0.88	0.27*	0.15, 0.49	<0.001
Elevated SBP	0.75	0.41, 1.36	0.76	0.47, 1.24	0.60	0.33, 1.09	0.130
Elevated DBP	0.86	0.46, 1.59	0.74	0.46, 1.19	0.56	0.29, 1.06	0.009
Elevated FPG	1.16	0.58, 2.34	0.70	0.34, 1.35	0.52	0.25, 1.06	0.057

Model adjusted for: age, sex, country of birth, smoking, physical activity level, income, education, body weight, season, energy, fibre, alcohol, magnesium, zinc.

Footnote: †Median 25OHD concentration and dietary calcium intake in each tertile; *significant as compared to the reference category.

Appendix R Awards, funding and presentations

R.1 Awards

Year	Organisation	Award
2015	Mark Liveris seminar	Best poster presentation

R.2 Funding

Year	Organisation	Award
2014-2017	Curtin University	Curtin University Postgraduate Scholarship (CUPS) & Curtin Research Scholarship
2014	Nutrition Society of Australia	Student travel award – conference attendance (Hobart, Tasmania)
2016	Nutrition Society of Australia	Student travel award – conference attendance (Melbourne, Victoria)

R.3 Presentations

Year	Organisation	Presentation
2014	Mark Liveris seminar	Mark Liveris seminar – oral presentation
2014	Nutrition Society of Australia	Perth branch seminar – oral presentation
2014	Nutrition Society of Australia	National conference, Hobart, Tasmania – poster presentation
2015	Mark Liveris seminar	Mark Liveris seminar – oral presentation
2015	Nutrition Society of Australia	National conference, Wellington, New Zealand – poster presentation (presented by Assoc. Prof. Mario J Soares)
2016	Nutrition Society of Australia	Perth branch seminar – oral presentation
2016	Nutrition Society of Australia	National conference, Melbourne, Victoria – poster presentation