School of Public Health

Vitamin D Status and Endothelial Function

Ali Mahdi M Alyami

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Declaration

This thesis contains no material which has been accepted for the award of any other degree at Curtin University. It contains three published papers of which I am the primary author, which were used with permission from the publishers. To the best of my knowledge, the rest of this thesis contains no material published by any other author.

Signature: Ali Mahdi Alyami

Date: 25/7/2017

Acknowledgements

In Perth, the capital of Western Australia, I have spent four lovely years working every day to accomplish my PhD degree and to add another valuable dimension of understanding to the area in which I work. During this period, I have learned many research skills including human research planning, application for ethics approval and the recruitment of participants. It was a difficult endeavour, in which the assistance of supervisors, colleagues and laboratory staff was invaluable. I have learned safe laboratory procedures, methods of blood collection and processes for material ordering and storage.

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I have been responsible for the completion of the following research aspects of this thesis: screening and recruiting participants, blood collection and storage, assisting in blood analysis, measuring endothelial function and DEXA measurements. However, this acknowledgement does not include other aspects of the work that will be mentioned later in this thesis and that are already acknowledged in the published manuscripts.

Abstract

Background:

Low 25(OH)D status is becoming increasingly prevalent worldwide and in Australia, as is Type II diabetes mellitus (T2DM) and cardiovascular disease (CVD). Endothelial dysfunction is central to the processes of both T2DM and CVD, and there is evidence from previous studies of an association between low 25(OH)D status and endothelial dysfunction. These studies indicate, but do not prove, a causative link between low circulating 25(OH)D status and endothelial dysfunction. A limited number of randomised controlled trials have been conducted in this area, and these have yielded conflicting outcomes.

Aim:

This thesis aimed to examine the interrelationships between vitamin D status, endothelial dysfunction, systemic inflammation and dyslipidaemia to shed more light on this important area. We proposed that cholecalciferol supplementation will improve endothelial function, thereby improving systemic inflammatory biomarkers and endothelial cell activation molecules. Also, we hypothesised that levels of circulating 25(OH)D <75 nmol/L will contribute to endothelial dysfunction and dyslipidaemia, but the exact 25(OH)D level will vary with ethnicity. The long-term effects could be slowing the progression of T2DM and CVD.

Methods:

We conducted a systematic review and two cross-sectional studies of the previous research in this area. The systematic review evaluated the previous randomised controlled trials (RCTs) that examined the relationship between cholecalciferol supplementation and one or more of: endothelial function, endothelial cell activation molecules and inflammatory biomarkers. This enabled us to make a more definite conclusion about the relationship between cholecalciferol supplementation, endothelial function and systemic inflammation.

The first cross-sectional study compared lipids profiles and endothelial cell activation molecules among three tertiles of 25(OH)D levels. One hundred and one asymptomatic male and female participants were recruited for this study, however only 83 participants with completed data were eligible for analysis. We controlled for age, gender, BMI, McAuley's index, season, medications and parathyroid hormone (PTH).

The second cross-sectional study compared circulating 25(OH)D levels and glucose-induced endothelial changes between two different ethnic groups, Australian men of European descent and Middle Eastern men. This was achieved through pulse contour analysis (PCA), which utilized finger photoplethysmography to generate a digital volume pulse (DVP) derived measure of arterial stiffness (SI) index and reflective index (RI) in each group. We examined asymptomatic participants, 21 Australian men and 17 Middle Eastern men. We controlled for age, ethnicity, fat mass, blood pressure, insulin sensitivity index, seasonal variations, heart rate, android/gynoid ratio, physical activity and seasonal variations.

Results:

In the systematic review, we found that circulating 25(OH)D levels of >50 nmol/L, which is the current cut-off value set for bone health, are probably sufficient for healthy endothelial function. We found evidence suggesting that cholecalciferol supplementation improves the endothelial function of diabetic and pre-diabetic patients when 25(OH)D levels are raised from <50 nmol/L to >50 nmol/L. There was also preliminary evidence that in hypertensive patients, cholecalciferol supplementation which increases 25(OH)D levels from <50 nmol/L to 62 nmol/L may reduce systemic inflammation. However, we found no evidence of an improvement in endothelial function or a decrease in inflammation among healthy subjects without diabetes, pre-diabetes or hypertension.

The first cross-sectional study revealed that average circulating 25(OH)D levels of 97 nmol/L were associated with lower levels of low density lipoprotein cholesterol (LDL-C) and total cholesterol (TC). Also, levels of circulating 25(OH)D > 66 nmol/L were

associated with lower circulating levels of triacylglycerol. However, high circulating 25(OH)D was linked to higher levels of soluble vascular cell adhesion molecules (sVCAM), which may indicate a level of inflammation in the endothelial cells. Possibly in response to this higher level of sVCAM, we found high circulating 25(OH)D linked to higher level of hepatocyte growth factor (HGF), which works as a protective or healing agent for the endothelial cells. This may support some reports of a J-shape association between 25(OH)D status and CVD.

The second cross-sectional study found that Australian men of European descent had the higher mean circulating 25(OH)D levels (60.9 nmol/L) and Middle Eastern men had the lower 25(OH)D levels (40.4 nmol/L). Australian men, with vitamin D sufficiency and better insulin sensitivity, demonstrated the most efficient endothelial function with a significant decrease in the adjusted mean of integrated area under the curve of reflective index (IAUC-RI). In contrast, Middle Eastern men who had vitamin D deficiency and higher insulin resistance showed less significant decrease in IAUC-RI. However, this result did not reach statistical significance, possibly because of the higher affinity Gc phenotype of the vitamin D binding protein (Vitamin DBP) in the Middle Eastern men, as they have darker skin pigmentation (1). This ethnic difference may not have allowed adequate evaluation of 25(OH)D bioavailability in the Middle Eastern men.

Conclusion:

Overall, we found no evidence to increase the cut-off for circulating 25(OH)D to > 75 nmol/L. Based on the limited evidence base available, the current cut-off level of > 50 nmol/L seems to be sufficient for proper endothelial function. However, more randomised controlled trials need to be performed to make this conclusion with certainty. Additionally, more research needs to be conducted to determine at what level circulating 25(OH)D may become harmful. There are contradictory results regarding the higher levels of 25(OH)D; one study found a beneficial effect on endothelial function as measured by flow mediated dilatation (FMD), whereas our first study showed a harmful

increase in sVCAM; a marker of endothelial dysfunction. Our systematic review also provided us with valuable insights on how to design future RCTs in the area.

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Abbreviations:

µg	micro grams
$1,25(OH)_2D$	1,25 dihydroxy vitamin D (calcitriol)
25(OH)D	25 hydroxy-vitamin D
AGES	The age, gen, environment and susceptibility
A/G ratio	Android/gynoid ratio
AI	Adequate Intake
AIx	Augmentation Index
AT1R	Angiotensin II-type 1 receptor
BIA	Bioelectrical impedance analysis
BMI	Body mass index
Ca	Calcium
Calcitriol	1,25 dihydroxy vitamin D
CI	Confidence interval
CRP	C-reactive protein
CVD	Cardiovascular disease
CYP2R1, CYP3A4 and CYP24A1	Cytochrome P450 2R1 (vitamin D 25 hydroxylase enzyme)
CYP27B1	Cytochrome P450 27B1 (1 α hydroxylase)
DBP	Diastolic blood pressure
DEXA	Dual energy x-ray absorptiometry
DVP	Digital volume pulse
DSM-BIA	Direct segmental multi-frequency bioelectrical impedance analysis method
EC	Endothelial cell
EDHF	Endothelial-derived hyperpolarising factor
ED	Endothelial dysfunction
EF	Endothelial function
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
F	Female
FGF23	Fibroblast-like growth factor-23
FMD	Flow-mediated dilation
Gc phenotype	Group specific-globulin phenotype
GTN	Glyceryl trinitrate
HADK2MAG-61K	Human adipokine magnetic bead panel 2
HCVD2MAG-67K	Human cardiovascular disease magnetic bead panel 2
HDL-C	High density lipoprotein cholesterol
HGF	Hepatocyte growth factor
HOMA-IR	Homeostatic model assessment- insulin resistance
hsCRP	high sensitivity C-reactive protein
HELENA	Healthy lifestyle in Europe by nutrition in adolescence

HGF	Hepatocyte growth factor
Hypovitaminosis D	Circulating 25(OH)D < 25 nmol/L
IAUC	Integrated area under the curve
IAUC-RI	Integrated area under the curve for reflective index
IAUC-SI	Integrated area under the curve for stiffness index
IL-1$^{\beta}$	Interleukin 1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-12	Interleukin-12
IL-23	Interleukin-23
INF-γ	Interferon γ
IPAQ	International Physical Activity Questionnaire
IR	Insulin resistance
ISI	Insulin sensitivity index
Kg	Kilogram
LASA	Longitudinal aging study amsterdam
LC-MS/MS	Liquid chromatography-tandem mass spectrophotometry
LDL-C	Low density lipoprotein cholesterol
LL-37	Cathelicidin gene transcription
M	Male
Maamu	Finnish migrant health and wellbeing study
MANCOVA	Multivariate ANCOVA
MAPK	Mitogen-activated protein kinase
mBCA	medical body composition analyzer
MCP-1	monocyte chemoattractant protein 1
ME	Middle Eastern
MED	Minimal erythema dose
MetS	Metabolic syndrome
MMP-9	Matrix metallopeptidase 9
MPO	myeloperoxidase
N	Number
NAD(P)H-oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NDL	Nutrient data laboratory
NFNAP	National food and nutrient analysis program
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHANES	National Health And Nutrition Examination Survey
NHMRC	National Health and Medical Council
NHS	New Hoorn study
nmol/L	Nano-moles per litre
NO	Nitric oxide
NRVs	Nutrient recommended values

O2	Oxygen
OGTT	Oral glucose tolerance test
ONOO	Peroxynitrite
PAI-1	Plasminogen activator inhibitor-1
PCA	Pulse contour analysis
PG12	Prostacyclin-12
PR	Pulse rate
PRISMA	The preferred reporting items for systematic reviews and meta-analyses
PROSPERO	International prospective Register for systematic reviews
PTH	Parathyroid hormone
PPT	Peak to peak Time
PT-2000	Digital volume pulse trace-2000 device
PWV	Pulse wave velocity
QUICKI	Quantitative insulin-sensitivity check index
RAS	Renin-angiotensin system
RCT	Randomised controlled trial
RDA	Recommended dietary allowances
REML	Restricted maximum likelihood
RH-PAT	Reactive hyperemia peripheral arterial tonometry
RI	Reflective index
RNS	Reactive nitrogen species
Rpm	Revolutions per minutes
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
SEs	Standard errors
sec	second(s) (time)
SI	Stiffness index
sICAM-1	soluble intracellular adhesion molecule-1
SMD	Standardised mean difference
sVCAM-1	Soluble vascular cell adhesion molecules-1
TAUC	Total area under the curve
T1	Tertile 1
T2	Tertile 2
T3	Tertile 3
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TF	Transfer function
TG	Triglycerides
TC	Total cholesterol
TLR	Toll-Like receptor
TLR-2 and TLR-4	Types of toll-like receptors

TNF-α	Tumour necrosis factor-alpha
UK	United Kingdom
U.S.	United States of America
US	Ultra sound
UVA	Ultraviolet sun light A
UVB	Ultraviolet sun light B
VDR	Vitamin D receptor
VD3	Cholecalciferol
VD2	Ergocalciferol
VDSP	Vitamin D standardization program
VEGF	Vascular endothelial growth factor
Vitamin D2	Ergocalciferol
Vitamin D3	Cholecalciferol
Vitamin DBP	Vitamin D binding protein
Vitamin D deficiency	Circulating 25(OH)D <50 nmol/L
WCC	White cell count
WHO	World Health Organization

Introduction

Vitamin D deficiency is becoming a worldwide issue, with studies revealing 25(OH)D levels of less than 50 nmol/L in many countries globally (2). The Australian Health Survey define levels of vitamin D sufficiency as 25(OH)D ≥ 50 nmol/L and total vitamin D deficiency as 25(OH)D < 50 nmol/L, these were set in regards to bone health. Specifically, deficiency is categorised into mild (25(OH)D = 30-49 nmol/L), moderate (25(OH)D = 13-29 nmol/L) and severe (25(OH)D < 13 nmol/L) (3). Due to a lack of sufficient data from many of the studies, we will refer to all 25(OH)D levels < 30 nmol/L as moderate to severe, and 25(OH)D levels < 25 nmol/L as hypovitaminosis D.

In Australia, 23% of adults have hypovitaminosis D despite the presence of substantial ambient sunshine (4, 5). Factors contributing to low vitamin D status include indoor living, advanced age, high coverage clothing, darker skin colour, seasonal variations and sun avoidance as a response to warnings about skin cancer (6). Additional factors include a lack of access to vitamin D supplements and a limited intake of foods which provide vitamin D; these include fatty fish, meat, liver, eggs and fortified milk and mushroom (5). One study showed that most Australian adults only acquire 5-10% of their vitamin D requirement through their diet (7).

Chronic non-communicable diseases, particularly cardiovascular disease (CVD) and Type 2 Diabetes Mellitus (T2DM), are continually becoming more widespread and together constitute the greatest disease burden worldwide. CVD and T2DM are currently responsible for more than half of the global mortality, and it is predicted that such chronic conditions will be the main cause of death worldwide by 2020 (8, 9). In 2014-15, in Australia 5.7% of men, 4.7% of women and 30.7% of all Australians above 75 years of age were CVD patients (10). In 2016, 1.086 million Australians were diagnosed with T2DM, and this is predicted to be 1,503,000 by 2030 (11, 12).

CVD and T2DM are pathological states in which inflammation and vascular pathology are predominant factors – this thesis will explore the probability that these factors are

partly dependent on vitamin D status and its modulation of endothelial function and insulin resistance. T2DM is an insidious disease which progresses over the years from a pre-diabetic state to a diabetic state in the absence of intervention; once established, it is generally a permanent chronic condition and often leads to cardiovascular compromise (13). For that reason, it is imperative that there be a focus on early detection and prevention of T2DM to lower the mortality rates related to CVD and diabetes (14).

The pathophysiology of T2DM can involve one or more of several genetic factors that have been associated with increased T2DM risk. Transcription factor 7-like 2 (TCF7L2) gene overexpression is one such genetic factor; this gene is highly associated with insulin secretion (15). In 2013, a meta-analysis study on TCF7L2 found that single nucleotide polymorphism of rs7903146, rs12255372, rs11196205 and rs290487 in TCF7L2 increased the risk of T2DM (16). T2DM is also linked to mutations of genes involved in mitochondrial protein synthesis, such as the 3243A>G mutation in the mitochondrial tRNA and the H324Q variant in the nuclear-encoded mitochondrial leucyl tRNA synthetase, which may lead to altered mitochondrial protein synthesis, thereby increasing the risk of glucose intolerance developing (17). Genotypes can also contribute to fasting plasma glucose (FPG) variation, which increases the risk of T2DM and CVD (18). These genotypes include mutations of loci which encode glucokinase (GCK), islet-specific glucose 6 phosphatase catalytic subunit-related protein (G6PC3) and melatonin receptor type 1B (MTNR1B) (18). In summary, mutations of genes involved in insulin secretion, glucose intolerance development and fasting plasma glucose variation are all implicated in the pathophysiology of T2DM.

T2DM and CVD are largely linked to obesity and the sedentary lifestyles increasingly engaged in by many people around the world (19). Higher fat mass allows greater storage of some fat soluble vitamins, including vitamin D, and so a low 25(OH)D status may be a consequence of that state (20). Vitamin D deficiency in this situation may contribute towards developing T2DM (21). For example, a decrease in insulin secretion from pancreatic cells via vitamin D receptor activation may be followed by increased

parathyroid hormone (PTH) and insulin resistance (IR) leading to T2DM (6, 22-24). However, the relationship between low 25(OH)D, IR and obesity is not clear yet. The most recent research studies did not support a role for vitamin D in preventing obesity, so further randomised controlled studies are required to explain this relationship (24, 25).

Vitamin D deficiency is suspected to be a risk factor for endothelial dysfunction, insulin resistance and metabolic syndrome (MetS) (21). Endothelial cells interpret and respond to blood-borne signals and haemodynamic variations (26); they synthesise and secrete products which are responsible for vasodilation and vasoconstriction (26). Therefore, endothelial dysfunction (ED) can cause various harmful processes to occur in the body and thereby seriously deplete the health status of the affected individual. Insulin resistance (IR) occurs when body cells become unable to maintain normal glucose and lipid homeostasis, which leads to a requirement for higher levels of insulin to maintain a normoglycaemic state (27, 28). IR is a sign of metabolic syndrome, which is a precursor to CVD and T2DM (11). ED and IR are both implicated in artherosclerosis and cardiovascular demise, which is not surprising considering the interdependent relationship between endothelial function and insulin production (29). ED hinders the movement of insulin across the capillaries, and conversely a lower insulin production alters the balance between vasodilators and vasoconstrictors and thus modifies endothelial function (29).

Atherosclerosis is an disease in which plaque (lipid-laden macrophage) accumulates in the sub-endothelial part of the walls of the arteries (30). Its early pathophysiology includes endothelial dysfunction (ED), which is greatly influenced by the renin-angiotensin system (RAS) (31). Activation of the angiotensin II-type 1 receptor (AT_1R) at the endothelial cells prompts vasoconstriction and neuro-humoral activation (31). It also contributes to over-release of reactive oxygen species, which leads to a decrease of nitric oxide (NO) availability and apoptosis of vascular cells, as well as an increase of the expression of the oxidised low density lipoprotein (LDL) receptor, adhesion molecules, chemotactic factors and pro-inflammatory cytokines (31). The reduction of NO

availability leads to vasoconstriction which increases the release of endothelin-1, thromboxanes and serotonin; this consequently causes proliferation and migration of vascular smooth muscle cells, eventually culminating in atherosclerosis (32).

There are vitamin D receptors (VDR) in most body cells; therefore, vitamin D plays a vital homeostatic role in the body. This role includes the regulation of bone metabolism and oxidative stress as well as the onset of inflammatory states which may lead to chronic disease (33). There is strong evidence that vitamin D has a direct positive effect on insulin release via the VDR (23). As a major body organ, endothelial cell plays substantial role in vitamin D metabolism. Active vitamin D may be secreted from endothelial cells in order to modulate the local effects which control inflammatory mechanisms and vasculature (34). $1,25(OH)_2D$ binding to VDR leads to the expression of multiple genes, which include vascular endothelial growth factor (VEGF), which is downregulated in individuals with T2DM (35, 36). Thus it is clear that endothelial VDR, and thus vitamin D, eventually play a vital role in glucose homeostasis in the blood, endothelial function, vascular integrity and blood pressure maintenance.

Hypovitaminosis D can lead to higher levels of parathyroid hormone (PTH), which in turn may lead to increased insulin resistance (6, 24, 37-40). It may also permit an acute-phase inflammatory response, which may be partly responsible for the increased risk of cardiovascular events in individuals with low vitamin D (41). Vitamin D is known to be an important anti-inflammatory and immunomodulatory molecule, which conducts autocrine and paracrine signalling in immune cells (42, 43). Vitamin D promotes endothelial function in many ways, which include the enhancement of blood pressure, the prevention of arterial calcification and the suppression of inflammatory states (11, 35, 44-51). However, further investigation is needed to explain vitamin D mediated endothelial pathophysiology in MetS. Vitamin D deficiency has not only been linked to CVD but also to autoimmune conditions and certain inflammatory conditions (43, 52). These include systemic lupus erythematosus, inflammatory bowel disease, scleroderma,

rheumatoid arthritis multiple sclerosis, immune-mediated thyroid disease and T1DM (52).

This thesis investigates the relationship between vitamin D status and endothelial function. Therefore, it involves the measurement of 25(OH)D levels and endothelial physiology. Vitamin D status will be measured via radioimmunoassay (DiaSorin). Endothelial function can be evaluated by examining the functioning of either coronary arteries or peripheral arteries (26). The gold standard technique involves using both intracoronary agonist infusion and quantitative angiography, which is too invasive for the purpose of this research (53). Less invasive techniques measure endothelial function through major peripheral arteries. These techniques include flow-mediated dilation (FMD), augmentation index (AIx), central pulse wave velocity (PWV) and pulse contour analysis (PCA) (54). However, the simple technique of PCA is gaining credibility (55).

PCA was used in this research because our laboratory has sufficient experience in this technique, it is simple and can be performed by one operator (55). The concept of the PCA technique depends on generating a digital volume pulse (DVP) through employing finger photoplethysmography to measure stiffness index (SI) and reflective index (RI) (55). SI measures the large arteries' stiffness while RI measures the small arterioles' vascular tone (55). The results of the pulse trace system analysis of DVP are strongly correlated with the central augmentation index (AI) and pulse wave velocity (PWV) (55). PCA produces highly reproducible PWV results within the same participant (55). However, a weakness of PCA method is that the SI and RI indices calculated from the PWV are influenced by cardiovascular properties such as those that occur naturally in aging, and may also be different in participants with uncommon waveforms (56). The pulse amplitude is vulnerable to several confounding factors, however the amplitude does not affect the PWV and therefore the SI and RI, making this weakness of the PCA method inapplicable to the measurements taken in our study (56).

The increasing incidence of T2DM and CVD in conjunction with lower vitamin D status among Australian adults led us to evaluate previous studies linking vitamin D to endothelial function to attain a conclusion about their relationship. This thesis aimed to find the optimal cut off for 25(OH)D that would benefit endothelial health; this is done by systematically reviewing all randomised controlled trials of supplemental cholecalciferol. This thesis also includes two clinical studies: one to evaluate the association between tertiles of 25(OH)D and endothelial function among Australian adults; and another examined the differences in endothelial function between Middle Eastern and European Australian men, and the potential contribution of 25(OH)D.

Chapter 1:

Literature review

Chapter 1: Literature review

1 An overview of vitamin D

1.1 Vitamin D: forms, sources and requirements

Vitamin D supplementation as cod liver oil was first discovered as a effective treatment for rickets and osteomalacia in the 1930s (57). Current ongoing research has demonstrated that vitamin D acts in various classical and non-classical capacities, including as a prohormone, hormone, and cell signalling molecule for several pathways (58, 59). The various functions of vitamin D both in its capacity as a hormone and factor in various additional pathways have been elucidated following the identification of Vitamin D2 (ergocalciferol) in 1931 and subsequent discovery of vitamin D3 (cholecalciferol) in 1936 (58).

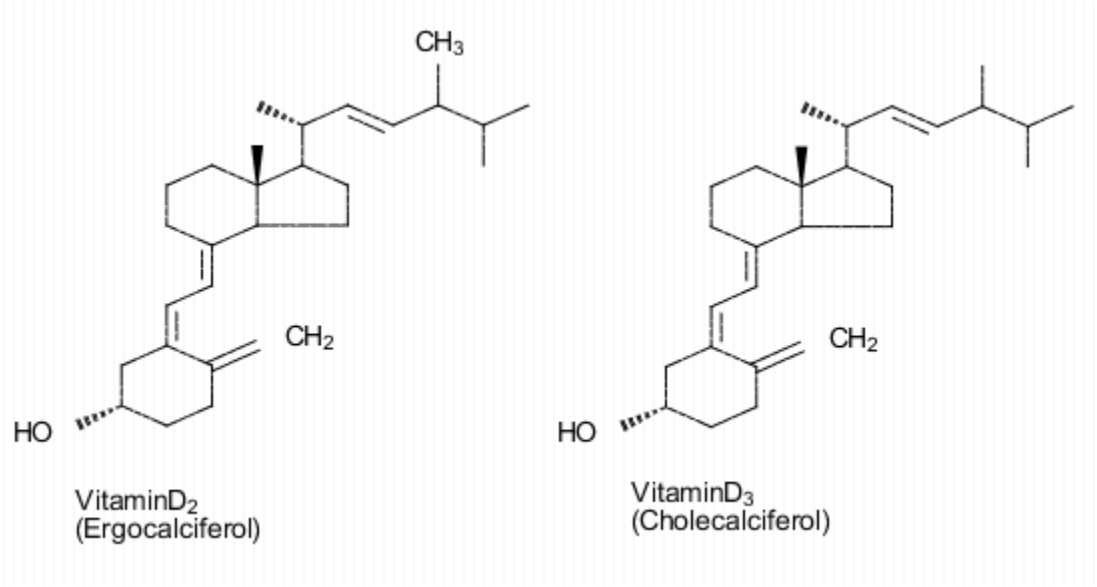

Figure 1: Vitamin D2 and Vitamin D3 chemical structure.

There are two ways in which the human body can obtain vitamin D: the conversion of 7-dehydrocholestrol in the skin to cholecalciferol (the most 25(OH)D source (5)); or via appropriate dietary intake according to the recommended daily intake (Figure 2) (59). Vitamin D is found in many forms throughout nature, however vitamin D2 (ergocalciferol, found in fungi) and vitamin D3 (cholecalciferol, found in mammals) are well-researched forms (Figure 1) (60). Vitamin D2 is formed following the conversion of

fungal sterol ergosterol after exposure to ultraviolet irradiation in plants (60). Meanwhile, vitamin D3 is the main product of conversion of 7-dehydrocholesterol in human and animals following exposure of melanocytes within the skin to ultraviolet sunlight (60). Vitamin D will be used in this thesis referring to both vitamin D2 and vitamin D3, unless otherwise stated. Neither vitamin D2 and vitamin D3 are active and require two hydroxylation reactions in the liver and kidney in order to produce the active form (59). The first reaction occurs in the hepatocytes wherein vitamin D hydroxylase transforms calciferol to 25 hydroxy vitamin D (25(OH)D). Cytochrome P450 2 R1 (CYP2R1) is mostly responsible for this reaction (59). Subsequently, 25(OH)D is converted to 1,25 dehydroxy vitamin D (calcitriol), the active form, in the kidney by 1-α-hydroxylase, with parathyroid hormone (PTH) mediated up-regulation, and fibroblast-like growth factor-23 (FGF23) mediated down-regulation (59).

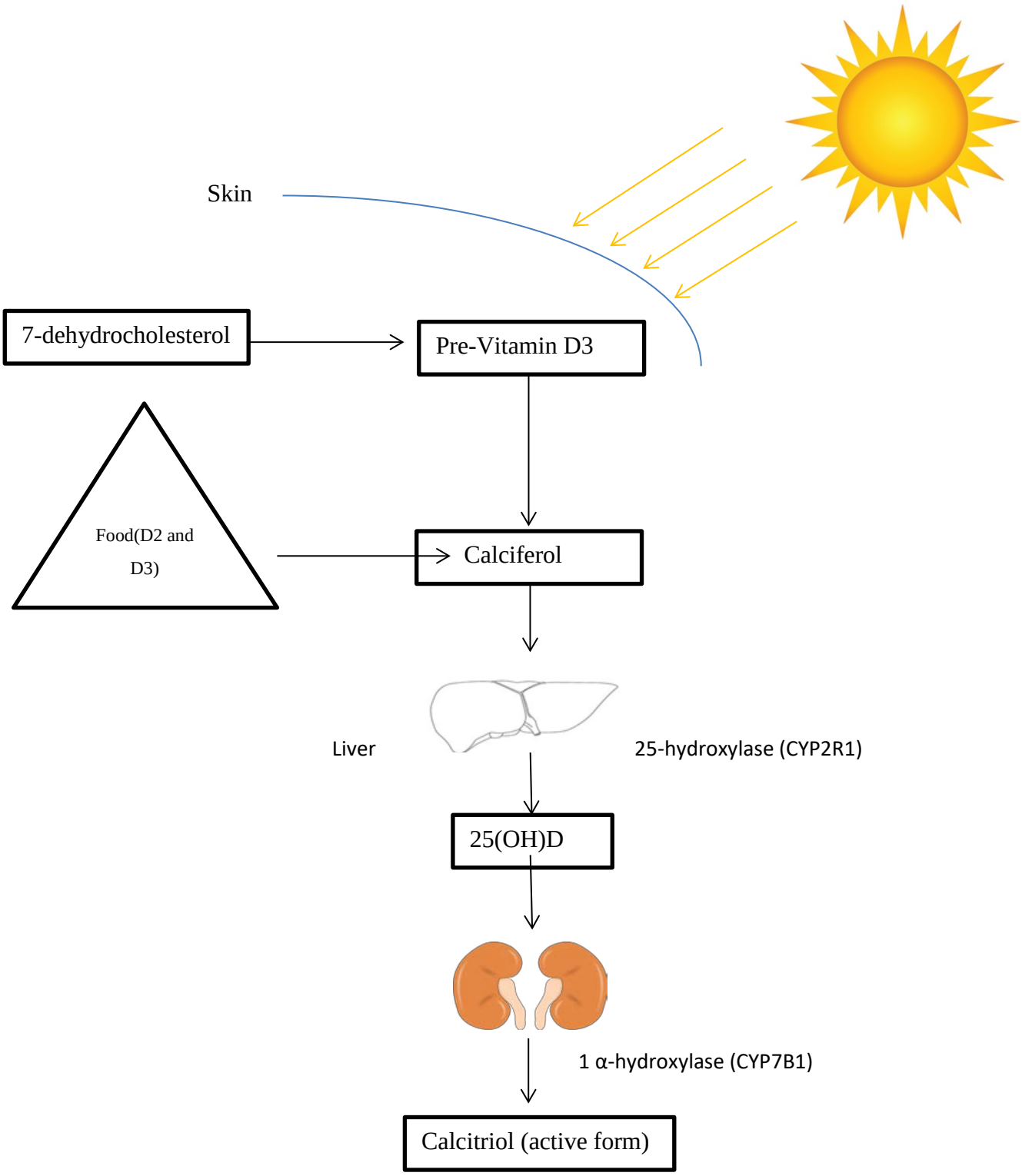

Figure 2: Vitamin D metabolism in the body. Vitamin D2: ergocalciferol, Vitamin D3: cholecalciferol, Calcitriol: active vitamin D (1-25 dihydroxy vitamin D).

The sunlight required to convert subdermal 7-dehydrocholestrol to inactive vitamin D is measured using minimal erythema dose (MED), which is the time required for the ultraviolet sun light B (UVB) to cause faint redness of the skin (5). However, erythema can occur after exposure to ultraviolet A (UVA) without synthesising vitamin D, which limits the accuracy of this index (5). The index time varies depending on the season, the angle of the sun light and proximity to the equator (5)(see Table 1).

Table 1: Period of time (h) needed to synthesise vitamin D through sun exposure in the middle of the day for moderately fair skin to achieve approximately one-third of MED.

Region and city	December-January 10 am or 2 pm (11 am or 3pm daylight saving time)	July-August 12 pm
Cairns	6-7	7
Townsville	5-7	11
Brisbane	6-7	11
Perth	5-6	15
Sydney	6-8	16
Adelaide	5-7	19
Melbourne	6-8	25
Hobart	7-9	29
Auckland	6-8	24
Christchurch	6-9	40†

MED: minimal erythemal dose, † in this city very little vitamin D made under the skin as there is limited UVB (5).

The traditional factors for poor vitamin status include illness or immobility (sedentary and indoor living), age related decline in skin synthesis, dress habits (covering the skin), skin colour and overall sun avoidance due to public health messages for skin cancer (61, 62). Not surprisingly the highest rate of vitamin D deficiency is seen in dark skinned,

veiled, pregnant mothers and the elderly and is more common in migrants to Australia as opposed to those of Caucasian origin and born in Australia (7, 62).

The secondary source of vitamin D is oral intake from food, fortified food, or supplements. There are only a few vitamin D containing food sources such as fatty fish (with high fat content such as salmon, sardine, herring and mackerel), meat (especially pig meat), liver, milk, eggs and mandatory fortified margarine (5, 63). Unfortunately, these foods do not contain a sufficient amount of vitamin D to meet current recommended levels (5). On the other hand, there are some products which are a valuable source of vitamin D such as fortified milk product with 200 IU (5 µg) of vitamin D per 250 mL serve, and mushrooms that have been treated by UV radiation that provide 800 IU (20 µg) of vitamin D2 per 100 g (5) (see table 2 for more vitamin D food content in the market).

In Australia, 19% of adults take a supplement which contains vitamin D and 5% of adults take a single vitamin D supplement (64). The use of a single vitamin D supplement, which contains 25 µg (1000 IU) of vitamin D, increases serum 25(OH)D by 10 nmol/L and significantly improves the likelihood of vitamin D sufficiency. Meanwhile, the use of supplements which contain vitamin D as well as other nutrients does not have such a great impact, because in these the vitamin D doses are lower and more varied; for each 1 µg of vitamin D that such a supplement contains, there is an increase in serum 25(OH)D by 0·41 nmol/L (65). Therefore, vitamin D status is only significantly impacted by the use of single vitamin D supplements in 5% of Australian adults (65). Australian adults are more likely to take a supplement containing vitamin D if they are female, more advanced in age, have higher level education, greater socio-economic status or higher levels of physical activity (65).

Table 2: Vitamin D amount in selected foods.

Food	Vitamin D, µg/100g	Vitamin D, IU/100g	Source of data
Spreadable butter	10	400	Label claim
Spreadable olive oil	10	400	Label claim
Cornflakes cereals	3.575	143	Label claim
Egg substitutes	1.6	64	Label claim
Malted drink powder	0.5	20	Label claim

Vitamin D intake through diet in Australia is poor. According to Pascoe et al. (2001), the average vitamin D intake among Australians is 1.2-2.6 µg/day (48 -104 IU/day) suggesting that for most Australian adults dietary intake only makes a small contribution of 20% to 50% of their daily vitamin D requirement (66, 67). Adequate daily intake for Australian adult (<50 years) is 5 µg/day (Nutrient recommended values (NRVs)), which is only a third of the more recent United States (U.S) recommended dietary allowance (RDA) for vitamin D, as seen in Table 3 (5, 67, 68).

Table 3: Comparison between vitamin D daily adequate intake (AI) for Australian adults and recommended dietary allowance (RDA) for US adults.

Gender	Age	AI	RDA
Men	19-30 years	5.0 µg (200 IU)	15.0 µg (600 IU)
	31-50 years	5.0 µg (200 IU)	15.0 µg (600 IU)
	51-70 years	10.0 µg (400 IU)	15.0 µg (600 IU)
	>70 years	15.0 µg (600 IU)	20.0 µg (800 IU)
Women	19-30 years	5.0 µg (200 IU)	15.0 µg (600 IU)
	31-50 years	5.0 µg (200 IU)	15.0 µg (600 IU)
	51-70 years	10.0 µg (400 IU)	15.0 µg (600 IU)
	>70 years	15.0 µg (600 IU)	20.0 µg (800 IU)

National Health and Medical Research Council (NHMRC) and National Institutes of Health (67, 68).

1.2 The physiological roles of vitamin D in the body

Vitamin D has a plethora of physiological roles within the human body as a hormone and as a cellular messenger. The most well recognised and accepted function of vitamin D is its implications in calcium and phosphate homeostasis and metabolism through augmentation of calcium absorption (59). Accordingly, pathological states associated with calcium and phosphorus homeostasis have been well analyses within the literature. Numerous studies demonstrate increasingly roles of vitamin D in infectious disease, autoimmunity, cardiovascular disease, musculoskeletal conditions, diabetes mellitus (both type 1 and type 2), several malignancies, neurocognitive decline and various pregnancy outcomes (69). This review will focus on the less well known and enigmatic actions of vitamin D. Interestingly, vitamin D receptors (VDRs), a member of the nuclear receptor superfamily, are ubiquitously present throughout body tissues that are not involved in calcium and phosphate homeostasis (33). Genetic studies conducted by Zella et al (2008) also showed that calcitriol regulates hundreds of genes potentially

representing up to 5% of the entire genome (70). Notwithstanding this, the significance of this control mechanism is not understood.

Vitamin D mediated immune modulation is particularly of interest within the research literature. The nexus between vitamin D and inflammation lies in the inverse relationship between circulating 25(OH)D and inflammatory biomarker levels (71). Indeed, controversy remains as to which of the variables is causative of lowering the other (71). Nonetheless, vitamin D receptors are present on an array of immune cells, including antigen presenting cells, T and B lymphocytes and monocytes (72). Evidence supports a multifactorial role for vitamin D in immune function such as the promotion of tolerogenic status, attenuation of autoimmune mediated effects in the context of autoimmune disease, and monocyte/macrophage synthesis of $1,25(OH)_2D$ as an integral component of innate immune function (72, 73). Vitamin D mediated modulation of T-lymphocytes, B-lymphocyte and macrophage/monocyte populations highlights an important role for vitamin D in the appropriate augmentation of inflammatory states (71).

Vitamin D also alters CD4+ T-cell differentiation and promotes monocyte maturation (26). Liu et al. (2006) demonstrated the role of monocyte/macrophage synthesis through gene analyses that demonstrated toll-like receptor (TLR) 2/1 induced macrophage expression of CYP27B1 and VDR. A series of studies indicated that cathelicidin gene transcription (LL-37), which encodes proteins involved in intracellular bacterial killing pathways, is stimulated directly by 1,25(OH)2D-VDR interaction (74-76). Several studies using human cultures and gene analyses comprehensively demonstrate a significant role of vitamin D in the antimicrobial activity of monocytes, and therefore the importance of sufficient vitamin D status in humans (73, 77). The role of vitamin D in endothelial function and the inflammatory modulatory effects of vitamin D on endothelial function are elucidated further in "*Vitamin D and endothelial function" (a review paper (26)) end of this chapter).*

Absorption and metabolism of vitamin D

Vitamin D is absorbed at the gastrointestinal tract luminal lining. The former process occurs when dietary intake containing vitamin D is processed within the human gut, resulting in the degradation of food components and absorption of 25(OH)D (e.g. from meat). Based on the lipid soluble nature of these compounds, the molecules are absorbed at the level of the small intestine alongside other lipid-soluble nutrients. Indeed, it has been suggested that vitamin D absorption relies on the presence of lipid-rich foods in order to provoke bile and pancreatic lipase release into the duodenum and fat emulsification (78, 79). Subsequently, emulsified fats and vitamin D can travel into the enterocytes of the small intestinal tract packaged as micelles (59). Once micelles cross the enterocyte, vitamin D is packaged in chylomicrons alongside cholesterol, triacylglycerols, other lipid-soluble substances and apolipoproteins that eventually enter the systemic circuit via the lymphatic network (59). This highlights the increased efficiency of vitamin D absorption and metabolism when consumed with fat-containing foods (80, 81). This is supported by studies demonstrating that pancreatic insufficiency and impaired bile release hinders vitamin D absorption at the gastrointestinal interface (82-84). Nonetheless, no optimal lipid intake that correlates with peak vitamin D absorption has been determined, and a recent review did not find sufficient data that a high fat content meal significantly increases cholecalciferol bioavailability (63). Additionally, a minimal amount of absorbed vitamin D also travels directly to the liver with monosaccharides and amino acids (59).

Adipose tissue postprandial uptake has been depicted rapidly as plasma 25(OH)D decreased (63, 85). This highlights the relationship between increased adiposity and reduced calcitriol (59). This is significant for people who do not expose themselves to the sunshine often enough, for whom food is the main source of vitamin D; this may include people who are elderly or hospitalised (63).

Metabolism of prohormone vitamin D to its active form (calcitriol) occurs through two hydroxylation steps, the first of which occurs in the liver and the second in the kidney.

These hydroxylation steps utilise different enzymes. Liver based hydroxylation to 25(OH)D is catalysed by at least one CYP enzyme (86). The broader regulatory mechanisms of this enzymatic hydroxylation process are largely unknown, however it is likely that the specific enzyme responsible is CYP2R1, as has been illustrated in multiple in vitro and genome studies (86-88). CYP2R1 has been shown to have greater 25-hydroxylase activity than other enzymes proposed to demonstrate such activity, including CYP3A4 and CYP27A1 (88). Other enzymes, including 7-dehydocholesterol reductase and CYP24A1, as well as Gc protein (also known as vitamin D-binding protein (DBP)) have been reported to affect serum 25(OH)D levels (1, 59, 89).

Vitamin DBP bound 25(OH)D travels to the kidney via the blood before it the molecule undergoes its second hydroxylation by 1α-hydroxylase (CYP27B1) to form calcitriol the active form of vitamin D (90). Serum calcium and phosphate levels are instrumental in the regulation of this final rate-limiting step, both of which are regulated upstream primarily by PTH synthesis and secretion and to a lesser degree fibroblast-like growth factor-23 (FGF23) phosphaturic activity (59). The former acts to decrease renal expression of sodium/phosphate transporters, ultimately leading to a reduction in serum calcitriol (59). Meanwhile, calcium sensitive PTH secretion from the parathyroid gland leads to increased expression of 1α-hydroxylase (CYP27B1) consequently increasing calcitriol formation to facilitate the end effect of enhanced vitamin D dependent intestinal calcium absorption. This demonstrates the significance of vitamin D sufficiency in relation to calcium, and therefore bone, homeostasis.

Storage of vitamin D

Non-specific vitamin D stores are located within the adipose tissue, most likely due to the lipophilic nature of vitamin D, however more specific mechanisms of vitamin D mobilisation and storage states and the physiological ways in which they are favoured remain unknown (91-93). As will be discussed throughout this review, the implications of vitamin D storage in adipose tissue remains an important nutritional consideration as the

prevalence of obesity rises in Australia (94). Moreover, it has been highlighted that increased adiposity and therefore vitamin D storage may reduce the availability of the compound such that larger dietary or supplemental 25(OH)D is required to achieve appropriate and homeostatic levels (5).

Vitamin D toxicity

Vitamin D toxicity occurs at 150-200 nmol/L of vitamin D in the blood (5) Hypercalcaemia and hypercalcuria are the most concerning outcomes of vitamin D toxicity in human. The only aetiology of vitamin D toxicity is hyper-supplementation with oral or intravenous vitamin D (1). Daily supplementation with more than 50,000 IU per day elevates plasma vitamin D levels to approximately 374 nmol/L in a healthy individual, while lower doses of around 10,000 IU a day over months will not cause such toxicity (95). Intramuscular vitamin D injections of 100,000 -150,000 IU may be beneficial for patients with poor absorption, however intramuscular preparations are not yet available in Australia (5). Interestingly, hypercalcemia may occur at levels above 140 nmol/L and hypercalcaemia is not seen until 25(OH)D in the blood reaches 220 nmol/L, and is often not reported until levels exceed 500 nmol/L (96).

1.3 Vitamin D epidemiology in Australian and worldwide populations

Vitamin D deficiency is a major global issue with total deficiency levels of 25(OH)D < 50 nmol/L being widely observed around the globe (2). The most at-risk regions for vitamin D deficiency are more likely to be observed in Southern Asia and Middle Eastern regions (2). The risk factors for lower vitamin D levels include: old age and associated skin changes, gender, sunlight angle, season, skin colour (darker skin synthesises less vitamin D less through sun exposure), vitamin D availability in the diet and vitamin D fortification (2). This section explores the epidemiology of vitamin D deficiency in Australia and other regions of the world.

The limits set by the Australian Health Survey define levels of vitamin D sufficiency and deficiency by the circulating 25(OH)D that can be measured in the blood. Vitamin D

sufficiency is defined as 25(OH)D ≥ 50 nmol/L and total vitamin D deficiency as 25(OH)D < 50 nmol/L. Specifically, deficiency is categorised into mild (25(OH)D = 30-49 nmol/L), moderate (25(OH)D = 13-29 nmol/L) and severe (25(OH)D < 13 nmol/L) (3). For the purposes of this review, we will refer to all 25(OH)D levels < 30 nmol/L as moderate to severe, due to a lack of sufficient data from many of the studies as to the specific levels of each participant. This literature review includes many studies which have used the previous definition and cut-off for hypovitaminosis D, which was set at circulating 25(OH)D < 25 nmol/L, therefore we will reference hypovitaminosis D where it most accurately represents the data to do so (see table 4).

Table 4: Classifications of vitamin D status using plasma 25(OH)D levels.

Term	Range
Vitamin D sufficiency	25(OH)D ≥ 50 nmol/L
Total vitamin D deficiency	25(OH)D < 50 nmol/L
Mild vitamin D deficiency	25(OH)D = 30 – 49.9 nmol/L
Moderate vitamin D deficiency	25(OH)D = 13 – 29.9 nmol/L
Severe vitamin D deficiency	25(OH)D < 13 nmol/L
Moderate to severe vitamin D deficiency	25(OH)D < 30 nmol/L
Hypovitaminosis D	25(OH)D < 25 nmol/L

The studies included in this review used one or both of two different methods of measuring circulating 25(OH)D. Radioimmunoassay has been commonly used worldwide, but liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a newer method that is becoming more reliable in measuring 25(OH)D. A recent study exploring the methods of vitamin D quantifications claimed that radioimmunoassay slightly overestimated vitamin D deficiency by up to 3% (97) when compared with LC-MS/MS.

Australia and New Zealand

In Australia, 23% of adults and an even greater proportion of elderly people have vitamin D deficiency (4, 5). As many as 50% of women may experience vitamin D deficiency during winter (5). A 2007 study in Australia compared the levels of serum vitamin D in winter and spring to those in summer and autumn. In winter and spring, deficiency was

40.5% in southeast Queensland, 37.4% in Geelong and 67.3% in Tasmania (98). They also found hypovitaminosis D to be 7.1 % in southeast Queensland, 7.9% in Geelong and 13% in Tasmania (98). In summer and autumn, deficiency was 18% in southeast Queensland, 10% in Geelong and 32% in Tasmania (98). A study of aged-care facilities in northern Sydney found a high prevalence of moderate to severe vitamin D deficiency (circulating 25(OH)D < 28 nmol/L) in the elderly residents, specifically in 68% of men and 86% of women, with a mean circulating 25(OH)D of 17 nmol/L overall (99). A case study conducted in Royal Hobart Hospital in Tasmania found that 67% of elderly patients admitted with a hip fracture had moderate to severe vitamin D deficiency (25(OH)D <28 nmol/L) (100). A cross-sectional study of elderly Tasmanian people living in the community revealed that 17% had moderate to severe vitamin D deficiency (<28 nmol/L) (101).

A New Zealand study measured the levels of plasma vitamin D in elderly women living independently, and found that in summer 26% had vitamin D deficiency levels of 25(OH)D < 40 nmol/L, while in winter this proportion increased to 69% (102). An Auckland study revealed vitamin D deficiency in 49% of women and 9% of men (103).

Asia

In the Southern Asian regions, vitamin D deficiency precipitates seemingly less severe effects. Postmenopausal women in Indonesia and Malaysia have a mean mild vitamin D deficiency (25(OH)D = 48 nmol/L) (104). In India, vitamin D deficiency has been demonstrated in several populations including neonates, children, pregnant women and healthy adults (105-109). Furthermore, vitamin D deficiency levels have been demonstrated among Pakistanis, although most alarming is the hypovitaminosis D among infants (110, 111). Bangladeshi and Sri Lankan populations have been shown to have mild to moderate vitamin D deficiency (hypovitaminosis D according to their cut-off which is set at 37.5 nmol/L), particularly among women of all ages and low income groups (112, 113). In China and Mongolia, total vitamin D deficiency rates are high and this is a major health issue alongside deficient calcium intake, leading to elevated rickets

prevalence among Chinese and Mongolian children (114). Moreover, a study conducted in China demonstrated 89% of adolescent girls had total vitamin D deficiency (115), and 48% of the elderly had hypovitaminosis D (116). Japanese studies illustrate vitamin D levels to be higher than in neighbouring regions such as China and India where moderate to severe vitamin D deficiency is common, again more prevalent amongst inactive elderly and women under 30 years old (117). Individual migrants of Asian heritage, regardless of gender or age, seem to be particularly vulnerable to vitamin D deficiency (118, 119).

South America

In South America, vitamin D deficiency prevalence varies within the same country. In Argentina, northern population have a mean 25(OH)D of 52 nmol/L, while southern areas have a mean mild deficiency of 36 nmol/L (120). An epidemiological study of vitamin D status and osteoporotic women conducted in Mexico, Chile and Brazil with a cut of 75 nmol/L demonstrated lower mean values in Mexico (65.5 nmol/L) compared to Chile (75.5 nmol/L) and Brazil (81.5 nmol/L) (121).

North America

The National Health and Nutrition Examination Survey (NHANES) from 2001 to 2006 demonstrated approximately 8% prevalence of moderate to severe vitamin D deficiency, and a further 24% have mild vitamin D deficiency (122). These results were determined by radioimmunoassay (DiaSorin), which may have slightly overestimated deficiency by up to 3% (97). Specifically, moderate to severe vitamin D deficiency was found to be highest among the elderly population aged >70 years old. Moreover, differences between ethnic groups and gender remained persistent (2). For example, black males had higher rates of moderate to severe deficiency compared to white and Hispanic males, while moderate to severe vitamin D deficiency was higher in women living beyond 70 years old compared to men (122).

More recent data from the NHANES (2007 to 2010) measured circulating 25(OH)D levels using a liquid chromatography–tandem mass spectrometry (LC-MS/MS),

calculating the sum of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in plasma (97). This study found that the prevalence of 25(OH)D levels <40 nmol/L is around 16% in all people over the age of one year (97). Increased vitamin D supplementation, awareness of vitamin D health benefits and the new technique for measuring circulating 25(OH)D may have all played a role in this improvement.

Canada

Canadian epidemiological studies demonstrate striking similarities to the North American prevalence of vitamin D deficiency. An observational study evaluating women aged 18 to 35 years old found that in winter, 21% of white women, 32% of non-white women and 25% of black women had serum 25(OH)D of <40 nmol/L (123). Nevertheless, a seasonal study performed on a population of elderly (>83 years) residing in a long-term care facility found that moderate to severe vitamin D deficiency was 9% during fall and 18% during winter (124). This seasonal variation is expected, however the lower overall rates among the elderly population contrasts the higher rate of moderate to severe vitamin D deficiency in the younger Canadian populations, as well as the neighbouring North American context. Furthermore, the vitamin D deficiency in the elderly populations increased from 38% during fall to 60% during spring (124).

Europe

In Europe, the most recent reports of combined population studies using LC-MS/MS, including over 55,000 adults and children, found that have 40.4% of individuals have total vitamin D deficiency, of which 13% have moderate to severe deficiency (125). It was found across nine European countries – Greece, Spain, Italy, Hungary, Austria, France, Belgium, Germany and Sweden – that 27% have mild vitamin D deficiency (defined in this study as 25(OH)D = 27.5-49.99 nmol/L) while 15% of the populations have moderate to severe deficiency (here defined as < 27.5 nmol/L), according to a study conducted by Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) (126, 127). Specifically, one study in a German paediatric population revealed that 44.5% had total vitamin D deficiency, of which 6% had hypovitaminosis D (125, 128). Similar

values were collected in an adult study in Germany where it was found that 54.5% have total vitamin D deficiency, of which 4.2% have hypovitaminosis D (125, 129, 130).

In the United Kingdom (UK), adult studies using Vitamin D Standardization Program (VDSP) Calibration found that 57.9% have total vitamin D deficiency and 16.8% of these have hypovitaminosis D (125, 131). In Ireland, cohort studies examining children from birth until five years of age using VDSP-LC-MS/MS found that 26.7% have total vitamin D deficiency and 1.6% of these have hypovitaminosis D (125, 132). Furthermore, another Irish population study using VDSP-LC-MS/MS depicted 43.7% with total vitamin D deficiency and 11.4% of the cohort with moderate to severe vitamin D deficiency (133).

In Iceland, the age, gene, environment and susceptibility (AGES) study used VDSP-Calibration to find that 33.6% have total vitamin D deficiency and 4.2% of these have hypovitaminosis D (125, 134). Meanwhile, two studies in Denmark using VDSP-Calibration found that 36.8% have total vitamin D deficiency and 2.6% of the population have hypovitaminosis D (135, 136). An additional Danish study using VDSP-Calibration discovered 23.6% had total vitamin D deficiency and 4.3% of the population had moderate to severe deficiency (137). In Norway, 76.1% were shown to have total vitamin D deficiency and 39.6% of these had hypovitaminosis D (125, 138). Another Norwegian study used VDSP-Calibration to find that only 18.6% of the population had total vitamin D deficiency and 0.3% of these had hypovitaminosis D (125, 139, 140). A third Norwegian study differs again, where the radioimmunoassay protocol for measuring 25(OH)D demonstrated that 26.9% have total vitamin D deficiency and 8.9% have moderate to severe vitamin D deficiency, and the LC-MS-MS method showed 6.6% to have mild vitamin D deficiency and a further 27.9% to have moderate to severe vitamin D deficiency (137).

A study in Finland used VDSP-Calibration to determine that 6.6% of the study population have total vitamin D deficiency and 0.4% of these have moderate to severe deficiency (125). A study in Hoorn, the Netherlands using radioimmunoassay method

found that in summer, 32% have total deficiency and 1.7% have moderate to severe deficiency, and in winter, 44.3% have total deficiency and 6.6% have moderate to severe deficiency (141). Another Dutch study (LASA: Longitudinal Aging Study Amsterdam) used VDSP-Calibration to find that 28.5% have total vitamin D deficiency and 2.4% of these have moderate to severe deficiency (125, 142). In Greece, 62.4% have total vitamin D deficiency and 2.2% of these have moderate to severe deficiency, as determined by VDSP-LC-MS/MS methods (125, 143). It is evident that throughout the majority of the European nations, the prevalence of total vitamin D deficiency and, of particular importance, moderate to severe vitamin D deficiency is relatively high.

Middle East

The Middle East experiences high UVB levels, however, vitamin D status is still low in most countries, as measured by radioimmunoassay. This could be a result of some cultural and religious practices, such the wearing of hijabs, veils and modest clothing, and hot weather in some countries. In the Kingdom of Saudi Arabia (KSA), as many as 80% of adolescent girls have hypovitaminosis D (144). In Riyadh KSA, a 2012 study found that 99.8% of children have moderate to severe vitamin D deficiency (25(OH)D < 30 nmol/L); specifically, 83% of 331 boys and girls had hypovitaminosis D (25(OH)D levels < 25 nmol/L), while 16.8 % had moderate vitamin D levels between 25 – 29.9 nmol/L (145). A recent study revealed mean mild and moderate deficiency levels of circulating 25(OH)D in Saudi boys and girls, 40 nmol/L and 27 nmol/L respectively (146). Similarly, in Saudi adults, the mean levels of circulating 25(OH)D reveal mild deficiency in both men and women, 40 nmol/L and 31 nmol/L respectively. Another study discovered that 83% of back pain patients have hypovitaminosis D (147). A cohort study of 3,475 Saudi adults showed a mean mild deficiency (25(OH)D = 35.5 nmol/L), however 36.1% of women and 48.8% of men had hypovitaminosis D (148).

Similarly, a Saudi cross-sectional study of healthy men found that 48.9% had hypovitaminosis D and 38.9% had mild to moderate deficiency, with circulating 25(OH)D levels between 25-50 nmol/L (149). Meanwhile, among adolescents, around

70% of Iranian girls and as many as 32% of Lebanese girls and 9-12% of Lebanese boys have hypovitaminosis D (150-152).

Conclusion

It is apparent that vitamin D deficiency is spread worldwide, and there are many factors contributing to this, such as skin colour and cultural, religious and lifestyle practices, including inactivity, wearing high coverage clothing for women, excessive use of sunscreen, eating diets poor in vitamin D, and living and working predominantly indoors. However, the level for determining vitamin D deficiency was set based on that needed for bone health (25(OH)D $\geq$ 50 nmol/L), which research indicates is less than the levels needed for extra-skeletal benefits. Thus, a new cut off needs to be determined to ensure people receive the other benefits of vitamin D as well, which would increase the prevalence of vitamin D deficiency around the whole world.

2 Endothelial Function

2.1 Endothelial function and dysfunction

Endothelial cells are a form of epithelium layer that lies sandwiched between the interior lumen of blood and lymphatic vessels and the vascular smooth muscle (45). Therefore, the endothelium forms the fluid-structure interface that facilitates fluid movement functions. The endothelium plays a vital role in the functions of autocrine, paracrine and endocrine signalling systems (153). The role of endothelial cells is to interpret and respond to blood-borne signals and haemodynamic alterations (26). Optimal endothelial function is the ability to respond to physical and chemical changes in the body by releasing many components that regulate smooth muscle cell proliferation, vascular tone, thromboresistance, cellular adhesion and vessel wall inflammation (154). The endothelium can act to synthesise and secrete its major vasodilatory products: nitric oxide (NO), endothelial-derived hyperpolarising factor (EDHF), and prostacyclin (PG12); and vasoconstrictive endothelin-1 (ET-1) to modulate vascular smooth muscle tone (26). This vasomotion of endothelial cells directly regulates vessel tone and diameter, which allows vascular tone to be measured as indirect indicator of endothelial function (154).

Specifically, endothelial-dependent vasomotion can be used as the endpoint in endothelial function assessment such as flow mediated dilation (FMD), augmentation index (AIx), pulse wave velocity (PWV) and pulse contour analysis (PCA). This is discussed in greater detail in the review paper (26).

Endothelial dysfunction (ED) is therefore characterised by several deleterious events that may compromise the health status of an individual both from a histopathological and gross physiological standpoint. Section 1.4.4 will consider the roles of vitamin D in endothelial physiology. Moreover, insulin resistance will be explored in relation to vitamin D status and the effects of hypovitaminosis, particularly as it aligns with the pathophysiological process of ED.

2.2 Endothelial function testing

Invasive methods:

Endothelial function has long been studied using many techniques. Endothelial physiology is most commonly evaluated by assessing either the coronary or the peripheral arteries (26). The gold standard technique for evaluation of endothelial dysfunction is the use of both intracoronary agonist infusion and quantitative angiography. This technique normally requires hospitalisation and is based on invasive procedures in which artery catheterisation is used to assess the endothelial-dependent vasodilation (53). Therefore, this method is unsuitable for use in most research studies as it requires a highly proficient practitioner to perform it and it is difficult to obtain ethics approval and permission from the participants. Alternative non-invasive methods have been developed that are more practicable than conventional methods (53).

Non-invasive methods:

The non-invasive methods that are commonly used in research include flow mediated dilation (FMD), Augmentation index (AIx), pulse wave velocity (PWV), reactive hyperemia peripheral arterial tonometry (RH-PAT) and pulse contour analysis (PCA) (47, 55, 155). These latter methods usually utilise the endothelium dependent pathway,

whereas FMD could use either endothelium dependent or independent pathway. Also, there are non-invasive methods of measuring systemic inflammatory biomarkers and/or endothelial cell activation molecules. In the following paragraphs, each method will be discussed separately.

2.2.1.1 Flow mediated dilation (FMD):

Studies evaluating peripheral arterial functioning now typically measure flow-mediated dilation (FMD) (54), which is a non-invasive and reproducible indicator of endothelial response to hemodynamic stress. FMD may be evaluated by the variation in the diameter of a peripheral artery through either the endothelium dependent pathway or the endothelium-independent pathway (26, 47). The endothelium dependent pathway will stimulate the release of NO from the endothelium which should vasodilate the peripheral arteries, whereas the endothelium-independent pathway bypasses the endothelium to relax the muscles, which should also vasodilate the peripheral arteries (47, 156). The endothelium-dependent pathway is usually activated by the administration of sheer stress, acetylcholine infusion or salbutamol inhalation, whereas the endothelium-independent pathway is initiated by administration of sub-lingual glyceryl trinitrate (GTN) (47). The change in diameter of a peripheral artery, usually the brachial artery, is then assessed using ultrasound (US) imaging (47, 54). High quality US images are essential for accurate analysis. Ultrasonographers require several months of hands-on training as well as continuous practice to ensure optimal quality and consistency of the data (47, 54, 156). US techniques are highly operator-dependent and at risk of investigator bias, and so simpler techniques may be used in research.

Reactive hyperemia peripheral arterial tonometry (RH-PAT):

RH-PAT measures EF by the application of a finger probe to track the changes in the digital volume that occur with pulse waves (157). This change is used to calculate the ratio by dividing the digital volume throughout reactive hyperemia by the baseline (157). RH-PAT is affordable, reproducible, operator independent and initial studies indicate it could have 80% sensitivity when compared with the gold standard technique (157, 158).

However, further studies are needed to assure the ability of RH-PAT to detect or predict patients with CVD (158).

Augmentation index (AIx):

AIx is a ratio calculation from the blood pressure waveform, this usually done through measuring aortic central AIx near the heart, which is invasive and not easy to evaluate, so a non-invasive technique of applanation tonometry is often used (159). This technique involves measuring the carotid artery AIx, which correlates well with aortic central AIx but is consistently lower than aortic AIx (160). Also, this technique needs a technician with a lot of experience, is not easy to apply to patients, and is not suitable for patients with major atherosclerotic plaques or calcified arteries (160). Thus, a generated transfer function (TF) technique has been invented to measure the central AIx using radial artery applanation. However, this technique's reliability is still questioned and insufficient to calculate the central AIx (160). These limitations of the TF technique have led to the use of the direct measurement of radial Aix using radial arterial tonometry, which showed substantial correlation with both invasively and non-invasively measured aortic central AIx values (161). However, the major limitation of AIx is that the timing of the arrival of the reflective pulse wave can be influenced by arterial stiffness, patient's height, left ventricular contractility, and arterial vasodilation properties (160).

Pulse wave velocity (PWV):

PWV is the time between two blood pressure wave form in different arterial sites (162). In PWV two electrocardiographs are simultaneously placed at two different surface landmarks and are used to determine the time it takes for the pulse wave to travel between these different artery sites, commonly the carotid and femoral arteries (162). The distance between these landmarks is used in conjunction with the pulse wave travel time to calculate the pulse wave velocity (162). A strength of PWV is that it is highly linked to endothelial function through the direct effects of endothelin-1 (ET-1) on large artery PWV and nitric oxide (NO) bioavailability on arterial stiffness (163). In detail, ET-1 regulates the endothelial smooth muscles cells which may cause arterial vasoconstriction,

while less NO increases arterial stiffness (163). The limitations of PWV include its need to be performed by a person with significant expertise, its reproducibility is not yet satisfactory and its validity is limited for patients with sinus arrhythmia (164).

Pulse contour analysis (PCA):

In PCA, the pulse trace system employs finger photoplethysmography to measure the amount of light absorbed by the haemoglobin thus producing a digital volume pulse (DVP) which facilitates stiffness index (SI) and reflective index (RI) measurements (55). The measured parameters from PCA are: stiffness index (SI) = time from first peak to second peak; (PPT) (sec)/height (m), which usually measures large arteries; and reflective index (RI) = ratio of the heights (amplitude) of wave B to wave A, which measures small arterioles (Figure 3)(55). While the former is a measure of large artery stiffness, RI is a measure of small artery vascular tone (Figure 3). Vasodilation leads to a smaller RI while vasoconstriction results in a rise RI values.

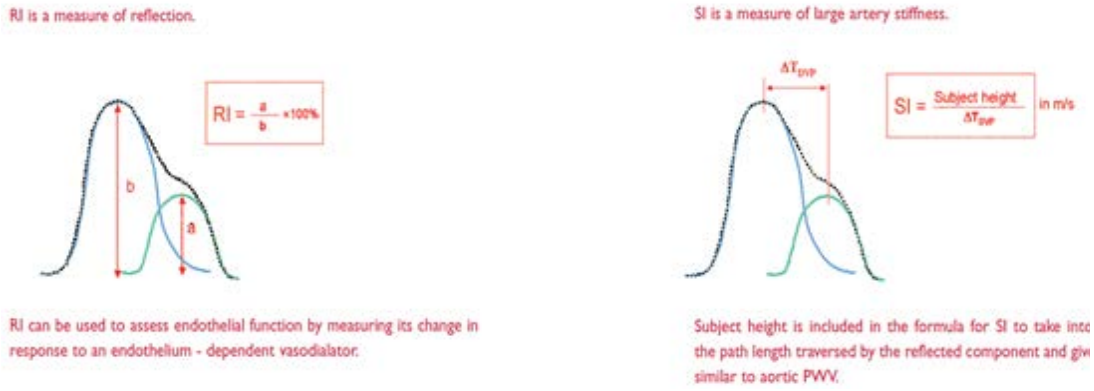

Figure 3: Pulse contour analysis (PCA) parameters, including refractive index (RI) and stiffness index (SI) for determining endothelial function (55).

Hence the pulse trace system analysis of DVP is simple and the results are strongly correlated with the augmentation index and central pulse wave velocity (55). PCA

produces highly reproducible PWV results, with a coefficient variation (CV) of 10.5% within the same participant (55). Another strength of this technique is that it can be conducted by one operator which made it easier for the primary researcher to work within the limited budget (55).

However, PCA results are more variable and thus less reliable (164). In particular, PCA measurement indices are affected by cardiovascular properties in older individuals and may also differ in individuals with uncommon waveforms (56). The pulse amplitude is also susceptible to the influences of respiration, an individual's cold fingers, sympathetic nervous system activity and factors contributing to local perfusion, however the pulse contour is mostly unaffected by the pulse amplitude (56). The SI and RI measurements rely mostly on the contour analysis which may not be affected by the pulse amplitude. Therefore, the susceptibility of the pulse amplitude is largely irrelevant for our study purposes. Our laboratory has extensive experience with PCA, including its cross-validation and its detection of changes in the reflective index (RI) as a response to nutrient manipulation (55).

Measurement of systemic inflammatory biomarkers and endothelial cell activation molecules:

EF can be evaluated through the measurement of systemic inflammatory biomarkers such as C-reactive protein (CRP), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα). EF can also be assessed by the levels of endothelial cell activation molecules such as soluble vascular cellular adhesion molecule (sVCAM), soluble intracellular adhesion molecule (sICAM), P-selectin and E-selectin (26, 47). These biomarkers are generally measured through blood tests. The relationships between these biomarkers and EF are discussed extensively in the review paper at the end of this chapter (26).

2.3 Endothelial dysfunction and its role in insulin resistance and metabolic syndrome

Insulin resistance (IR) is defined as the inability of body cells to maintain normal glucose and lipid homeostasis. Hence, higher levels of insulin are needed to retain a normoglycaemic state (27, 28). The results of insulin resistance are increased lipolysis in adipose tissue, impaired uptake of glucose by muscle and uninhibited gluconeogenesis (165). Insulin resistance is a common state that is associated with several predisposing factors such as genetic susceptibility, ageing, sedentary lifestyle and more increasingly, obesity (11). It is known that insulin resistance is strongly related to visceral obesity, hypertension, atherogenic dyslipidaemia and pro-thrombotic states. The presence of both IR and developing cardiovascular disease is collectively referred to as a 'metabolic syndrome' (MetS) (11). MetS is an indicator of developing CVD as well as possible progression to T2DM (11). Insulin resistance is central to cardiovascular disease, T2DM and therefore, MetS (11).

Nitric oxide is a soluble gas and vasoactive substance produced by the endothelial cells to regulate various systems and provides a strong measure of endothelial function. NO is synthesised from L-arginine in the endothelium by calcium-calmodulin-dependent endothelial NO synthase (eNOS) (45). NO is a critical messenger in blood pressure and vascular tone maintenance and plays broader roles in the immune, gastrointestinal, pulmonary and neurological systems (45). The endothelium is responsible for the NO secretion which plays a major role in regulating vessel diameter and therefore the arterial resistance throughout the vascular bed (46). Therefore, ED is a significant factor in the pathogenesis of atherosclerosis, hypertension and congestive cardiac failure (166). The endothelium is also responsible for the production of vasoconstrictors such as ET-1, which reduce arterial diameter and decreases resistance and pressure (48). Vascular tone relies on two systems: the production of NO by endothelial cells and the functioning of smooth muscles in the artery. Accordingly, endothelium-dependent vasodilation occurs as a response to pharmacological (e.g. salbutamol) and physical (e.g. shear stress) stimuli, which act through the endothelial cells to release NO (47). On the other hand,

endothelium-independent vasodilation bypasses endothelial cell function and depends on the smooth muscles in the artery (47); agents such as nitroglycerin function through this pathway.

ED leads to reduced nitric oxide (NO) dependent vascular activity, which is consistent with insulin-resistant states (11). Indeed, a plethora of evidence supports the role of insulin resistance and endothelial dysfunction in atherosclerosis and cardiovascular demise (29). The lack of endothelial derived NO secretion that occurs in the face of ED links the progression of ED and insulin resistance (29, 55). NO deficiency occurs due to decreased synthesis alone or combined with decreased secretion, in addition to the increase in consumption via high peripheral reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are by-products of metabolic disturbances in glucose and lipid metabolism (29). ED hampers insulin action by altering the transcapillary movement of insulin that facilitates the hormones access to target cells. Therefore, in conjunction with the potential reduction in microcirculatory blood flow to tissues the reduction in free insulin movement impairs the stimulation of glucose and lipid metabolism in response to insulin (29).

Insulin exerts its effects on endothelial cells via the production of NO, however it also controls endothelial function through an alternate pathway that produces endothelin-1 (ET-1), an endothelial derived vasoconstrictive agent (48). Therefore, an impairment of insulin secretion alters the balance between vasodilators and vasoconstrictors and subsequently alters endothelial function.

3 Vitamin D, endothelial function, insulin resistance, inflammation and metabolic syndrome

3.1 The correlation between vitamin D deficiency and endothelial function

Hypovitaminosis D is highly prevalent around the globe (2). It is acknowledged that the vitamin D receptor (VDR) is ubiquitously located in greater than 38 target tissues in which it exerts gene control of vital homeostatic functions such as bone metabolism, regulation of oxidative stress, and seemingly, the mitigation of chronic disease onset and inflammatory states (33). Endothelial cells are capable of synthesising and secreting $1,25(OH)_2D$ in order to modulate local effects, potentially controlling inflammatory mechanisms and their effect on vasculature (34). Evidence suggests that the active molecule(s) plays an active role in the pathways that control vascular mechanisms (167).

Vitamin D deficiency has been implicated in the complex interplay between endothelial dysfunction, insulin insensitivity and the development of metabolic syndrome (21). Some evidence suggests that hypovitaminosis D may decrease insulin secretion from pancreatic β cells and accelerate the development of insulin insufficiency and T2DM (22). Indeed, there is strong evidence that vitamin D has a direct effect on insulin release via the VDR (23). Ligand binding to VDR leads to the expression of several genes, including vascular endothelial growth factor (VEGF), which is down regulated in insulin insensitive individuals and type 2 diabetics (35, 36). Ahmad et al. (2006) demonstrated that VEGF-receptor mediated eNOS activation in endothelial cells leads to NO synthesis and may lead to vasodilation effects. Additionally, VDR gene knockout mice have been observed to experience impaired acetylcholine-induced aortic relaxation, with a reduction in eNOS expression and phospho-vasodilator-stimulated phosphoprotein levels (168, 169). Ni and Collegues (2014) showed that VDR knockout mice also experienced increased sensitivity to the hypertensive effects of angiotensin II in contrast to the controls, as well as increased hypertrophy-sensitive gene expression and type A and type B naturitic peptide levels. This study clearly suggests that endothelial VDR and therefore, vitamin D, plays a role in endothelial function, vascular integrity and blood pressure maintenance.

Increased PTH levels due to hypovitaminosis D are associated with increased insulin resistance (6, 24). Secondary hyperparathyroidism due to low vitamin D may also promote an acute-phase response potentially illustrating one additional reason for the increased risk of cardiovascular events in individuals with low vitamin D (41). Intact PTH levels have also been shown to predict insulin sensitivity in some models, including in studies conducted within our laboratory (37-40). Indeed, this demonstrates the need for further understanding of the implications of vitamin D mediated endothelial physiology in individuals with metabolic syndrome.

The relationship between ED, IR and vitamin D is complex; however, studies illustrate significant links between IR states such as T2DM and vitamin D sufficiency in promoting functional endothelial improvements. It is interesting to note that many type 2 diabetics have total vitamin D deficiency with associated endothelial dysfunction and depleted endothelial progenitor cells (170). Sugden et al (2008) conducted a double-blind, parallel group, placebo controlled randomised trial which demonstrated vitamin D supplementation improved 25(OH)D levels by 15.3 nmol/L and lead to a statistically significant enhancement in brachial FMD outcomes in diabetic participants with mild vitamin D deficiency (171). Moreover, the authors demonstrated a 14 mmHg decrease in systolic blood pressures in the treatment group in contrast to the placebo group, highlighting the endothelial and cardioprotective effects of vitamin D sufficiency in diabetic patients. Similar statistically significant functional endothelial improvements are reported in asymptomatic patients with vitamin D deficiency following replacement therapy (21).

There are various potential mechanisms in which vitamin D may enhance endothelial function. Firstly, vitamin D may lower blood pressure, which may be indirectly through renin suppression or directly through interaction with endothelial cells and the secretion of NO to decrease resistance of vessel walls (35, 44-48). Additionally, vitamin D sufficiency may aid in preventing arterial calcification which predisposes individuals to

adverse cardiovascular events, particularly those with insulin insensitivity and T2DM (11, 49). Watson et al (1997) found a correlation between low 25(OH)D and an increase in coronary calcification, which is pathogenically linked to the progression of atherosclerotic plaque formation; this is predictive of future coronary artery disease and myocardial infarction. Moreover, vitamin D has been observed to attenuate the inflammatory response. For instance, Sadeghi et al (2006) showed that vitamin D could quell the cellular actions of macrophages in order to reduce atheroma formation (51). Likewise, supplementation of vitamin D also suppressed tumour necrosis factor alpha (TNF-α), a powerful pro-inflammatory mediator, among a subset of chronic heart failure patients (50).Despite the aforementioned primary evidence, some studies dispute the role of vitamin D in endothelial physiology (172-183). This appears largely due to the failure to translate comparable outcomes from primary human, animal and in vitro studies to randomised control trial (RCT) format (26). This is explored in greater detail in the review of vitamin D and endothelial function paper (page 58).

3.2 The role of vitamin D and endothelial dysfunction in inflammation

As noted above, the literature illustrates a relationship between vitamin D and endothelial function (21, 26, 171). The link between hypovitaminosis D and progressive cardiovascular demise, including the progression of atherosclerosis, hypertension, peripheral artery disease, myocardial infarction, cerebrovascular disease and congestive cardiac failure beyond the scope of traditional cardiovascular risk factors further evidences the presence of an interaction between vitamin D and vascular endothelial function (167). The function of vitamin D in relation to endothelial function/dysfunction is not yet clear, although the endothelium has a well-studied and universally accepted pro-inflammatory role.

Vitamin D is a well-recognised immunomodulatory and anti-inflammatory molecule (42). Studies demonstrate that immune cells are capable of producing locally derived active vitamin D in order to conduct autocrine and paracrine signalling (43). Specifically, $1,25(OH)_2D$-producing immune cells express VDR and vitamin D3 metabolising enzyme

products such as 1α-, 25- and 24-hydroxylases (43). Furthermore, macrophages and dendritic cells constitutively express the VDR (184). These cells express 1α-hydroxylase and 25α-hydroxylase enabling the production of 25(OH)D and 1,25(OH2)D3 (37, 185).

Animal and in vitro studies have highlighted vitamin D's role in direct and indirect immunomodulation of T and B lymphocytes, in addition to antigen presenting cells such as macrophages and dendritic cells (43). Accordingly, it is suggested that vitamin D plays a pertinent role in both innate and adaptive immunity. Numerous studies outline a role of hypovitaminosis D in the relationship between low immunologic self-tolerance and autoimmunity (43, 186-188). Accordingly, the vitamin D deficient state has been implicated in systemic lupus erythematosus, inflammatory bowel disease, scleroderma, rheumatoid arthritis multiple sclerosis, immune-mediated thyroid disease and T1DM (52).

Moreover, the associations have been observed in inflammatory conditions, infections, malignancy, transplant rejection and cardiovascular demise (43). Vitamin D immunomodulation facilitates a fundamental immune system switch from Th1/Th17 to Th2/Treg response further explaining the influential role that vitamin D plays in autoimmune and inflammatory pathogenesis (43). This role has led to animal studies that suggest vitamin D may have therapeutic prospects for managing inflammatory and autoimmune pathology (43). For instance, a positive correlation between active vitamin D and augmented innate immune responses against infection has been demonstrated (189, 190). Notwithstanding the quantity of evidence, it is still unconfirmed whether or not vitamin D plays a role in immune function and its pathogenesis (42).

3.3 Obesity, vitamin D deficiency and progression to metabolic syndrome

Obesity represents a fundamental shift in functional metabolic demands. The western diet characterised by disproportionate consumption of energy dense foods containing high sodium, high sugar and high saturated fats and low intake of anti-oxidant plant sources and healthy fats is increasingly common (4, 191). Accordingly, energy excess and obesity

prevalence is growing rapidly, leading to proportionately greater adiposity, energy demands and therefore insulin requirements to ensure glucose uptake by muscle and fat cells. A multiplicity of studies link Vitamin D to the development of diabetes and cardiovascular disease, collectively referred to as metabolic syndrome, in which obesity is a primary risk factor (42). The aforementioned dietary patterns lead to adipose tissue macrophage infiltration and insulin resistance (42). Furthermore, Suganami et al (2007) propose that increased circulating saturated free fatty acids may interact as with macrophage and adipocyte TLR-2 and TLR-4 leading to NF-kB, c-jun N-terminal Kinase and p38 mitogen-activated protein kinase (MAPK) signalling cascade activation switching on pro-inflammatory gene transcription (192).

4 Conclusion

A plethora of epidemiological studies illustrate the high prevalence of low vitamin D levels around the globe. Evidence suggests that hypovitaminosis D correlates with endothelial dysfunction, increased inflammation, T2DM and CVD. This chapter assessed the nexus between one potential physiological driver, vitamin D and its role in the up and down stream functions of the vascular endothelium. The literature demonstrates that vitamin D carries out fundamental extra-skeletal functions including the modulation of endothelial cell physiology in order to regulate vascular structure and function.

Some aspects of the relationship between circulating 25(OH)D levels and endothelial function as described above have already been published in a review paper:
‘Vitamin D & endothelial function ‘by Alyami A, Soares MJ, Sherriff JL & Mamo JC.’ Ind. J Med. Res 2014 [Impact factor =2.0; cited by 11].
Please see the original paper next page. (Co-author permission signatures attached)

Review Article

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Vitamin D & endothelial function

A. Alyami, M.J. Soares, J.L. Sherriff & J.C. Mamo

Directorate of Nutrition Dietetics & Food Technology, School of Public Health, Curtin University, Perth, Australia

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There is increasing interest in the extra-skeletal roles of vitamin D for health and well-being. Poor vitamin D status has been associated with obesity, cardiovascular disease, type 2 diabetes and mental health. Endothelial dysfunction may underscore insulin resistance and hence predispose to both cardiovascular disease (CVD) and type 2 diabetes. The objective of this review was to gain an appreciation of the recent causative evidence linking vitamin D and endothelial function. the PubMed database was searched from 2009 to date. Key words used were vitamin D, supplementation, systemic inflammation, endothelium, endothelial dysfunction and humans. Selected articles were restricted to the English language and to randomized control trials (RCTs) of vitamin D supplementation with direct measures of endothelial function. Final inclusion was based on a quality rating ≥ 3, based on the Jadad score. Ten RCTs met these criteria and were summarized for their outcomes. Only two studies showed an improvement in flow mediated dilatation with vitamin D. Three other studies reported decreases in C-reactive protein, platelet activation inhibitor-1, tissue plasminogen activator and B type natriuretic peptide. Recent evidence from good quality RCTs did not support a beneficial effect of vitamin D on vascular reactivity. Future intervention studies may need to target a higher vitamin D status and longer duration to determine whether the vitamin has a regulatory role in endothelial function.

Key words Endothelial function - flow mediated dilatation - inflammation - obesity - supplementation - vitamin D

Vitamin D and health

Like many parts of the world including Australia, India faces the burden of obesity with a significant percentage (around 30-65%) of adult urban Indians diagnosed as overweight or obese or with abdominal obesity[1]. Interestingly both India and Australia have abundant milk supplies and plentiful sunshine, yet large sections of their population have lower than recommended dietary intakes of calcium (Ca)[2] and low vitamin D status[3-5]. Calcium and vitamin D have many potential roles in human physiology but are accepted mainly for their influence on bone health[6]. The evidence base that associates calcium intake and vitamin D status with obesity, cardiovascular disease, type 2 diabetes and more recently cognitive effects is growing. It ranges from the cellular to animal to human clinical and epidemiological investigations[7-10]. However, the balance of evidence that tilts either one or both nutrients towards an extra-skeletal health effect is yet to be reached. In this review we questioned whether

vitamin D status was causally linked to endothelial function. We present an overview of vascular function and commonly used methods to measure vascular dysfunction. All randomised controlled trials conducted in the recent past that have supplemented vitamin D have been collated to determine its putative effects on vascular function.

The vascular endothelium is a monolayer at the interface between blood and tissue. This pivotal role allows endothelial cells to detect and react to blood-borne signals and changes in haemodynamic forces. The vasodilatory impact of three endothelial cell (EC) products, nitric oxide (NO), endothelium-derived hyperpolarising factor (EDHF) and prostacyclin (PGI2), on the underlying smooth muscle cells is countered by the vasoconstrictor EC factor, endothelin-1 in the regulation of vascular tone[11]. In response to various stimuli including shear stress at the endothelial cell surface, NO can also diffuse towards the lumen and prevent both monocyte and platelet adhesion[11]. Thus NO's role reaches beyond vasodilation to encompass protection from inflammation and thrombosis after vascular injury. These roles are challenged by risk factors associated with cardiovascular disease.

Basal vasodilator tone is primarily controlled by the continual endothelium-dependent production of NO, however, the smooth muscle cells may not respond to this signal[12]. While activation of endothelial nitric oxide synthase (eNOS) is central to endothelial-dependent vasodilation, mechanisms supporting endothelial-independent vasodilation include the stimulation of phospholipaseA_2 activity[13]. In order to distinguish between EC and smooth muscle cell dysfunction, both endothelium dependant and independent systems need to be assessed[11].

Measurement of endothelial function

Endothelium function is usually measured by assessing either coronary arteries or peripheral arteries for vascular reactivity. The initial technique for evaluation of endothelial dysfunction was based on invasive procedures in which artery catheterization was required to assess endothelial dependent vasodilation[14]. Alternative non-invasive techniques have been developed that are more practicable than conventional methods. Peripheral artery studies usually focus on a phenomenon called flow-mediated dilation (FMD)[15]. FMD occurs when endothelial cells release NO in response to a shear stress[15,16]. Built on this principle, evaluation of FMD was developed using ultrasound imaging[15,17] that captured the change in the diameter of a peripheral artery (typically brachial artery)[17]. The measurement of FMD is considered the gold standard in measuring EF and is usually accomplished in response to sheer stress; acetylcholine infusion; salbutamol inhalation (reflecting the endothelium dependent pathway); or in response to sub-lingual glyceryl trinitrate (reflecting the endothelium independent pathway)[17]. Due to the high level of technical skill required in procuring and analysing ultrasound images, the use of other simpler techniques has gained popularity and credibility. Arterial applanation tonometry uses a sensitive probe applied to carotid and femoral arteries in the same subject to determine characteristics of the transmitted wave form[18]. Augmentation index (AI_x, ratio between the pulse pressure at the second systolic peak and the pulse pressure at the first systolic peak), is a derived variable that reflects endothelial function. Another validated system employs finger photo-plethysmography to produce a digital volume pulse analysis (DVP)[18]. The calculated parameters from this analysis are stiffness index (SI), a measure of large artery stiffness, and reflective index (RI) that signifies small artery vascular tone. Vasodilation leads to a smaller RI while vasoconstriction results in a rise in its value. Hence, the analysis of DVP is relatively simple and the results are strongly correlated with AI_x and central pulse wave velocity[17,18]. These non-invasive techniques have been used in conjunction with the appropriate pharmacological agent to measure both endothelium dependent and independent pathways of EF. Based on meal based stimuli, we demonstrated that the acute ingestion of calcium and vitamin D as part of a breakfast meal resulted in dose dependant changes in RI of Indian men based on a DVP analysis system[19].

Vitamin D, systemic and vascular inflammation

The link between systemic inflammation and vascular-specific inflammation from endothelial activation is well established[20]. There are numerous vascular markers of endothelial damage and their role in the pathophysiology of endothelial dysfunction is excellently covered elsewhere[11]. Hence, factors associated with thrombus formation and control, like plasma level of von Willebrand factor or plasminogen activator inhibitor (PAI-1) are a reflection of endothelial dysfunction[17]. Secondly, increment in the inflammatory markers such as C-reactive protein (CRP), cellular adhesion molecules (CAMs), vascular adhesion molecules, and P- or E-selectin have also been used to detect endothelial dysfunction[17]. Table I briefly

Table I. Some commonly used circulating biomarkers in studies of endothelial function

Biomarker	Stands for	The role in body	The relationship with endothelial function
D-dimer	A fibrin degradation product of cross-linked fibrin	Indicates the occurrence of thrombin generation and plasmin generation in the blood	Inverse[21]
sICAM-1	soluble intercellular adhesion molecule type 1	Helps leukocytes migrate across endothelial cells in the inflammatory state.	Inverse[22]
sVCAM-1	soluble vascular cell adhesion molecule-1	Assists lymphocytes, monocytes, eosinophil and basophils to adhere to vascular endothelium.	Inverse[22]
MPO	myeloperoxidase	Enzyme produced by the neutrophils; converts nitrite to nitrate; acts as a bactericidal agent, regulates the availability of nitric oxide in the blood; is associated with many chronic diseases.	Inverse[23]
P-selectin	P-selectin	Is an adhesion receptor for leukocytes on the surface of the endothelium.	Inverse[24]
E-selectin	E-selectin	Is an adhesion molecule for leukocytes in endothelial cells.	Inverse[22]
IL-6	interleukin 6	IL-6 triggers the production of collagenases and prostaglandins which reduce the pain threshold. Also stimulates T and B cells in their immune mechanisms.	Inverse[25]
IL-1β	interleukin- 1 beta	IL-6 beta is able to induce fever, anorexia and hypotension. May also control mycobacterial proliferation in the macrophage.	Inverse[26]
IL-12	interleukin -12	Improves the functioning of T-helper 1 while reducing T-helper 2, increases the number of T-helper 1 and natural killer (NK) cells, stimulation of T and NK cell cytotoxic activity, initiation of macrophages and anti-angiogenic.	Inverse[27]
Leptin	leptin	Besides its role in energy balance, leptin plays an important role in the immunity, inflammation and haematopoiesis. It improves the production of IL-2 and IFN-γ; modulates cytokines production from monocytes/macrophages.	Inverse[28]
HGF	hepatocyte growth factor	Regulates growth and morphogenesis of many cells in the body, including endothelial cells. Inhibits production of cytokines; enhances endothelial integrity and vascular barrier function.	Inverse[29]
hsCRP	high-sensitive C-reactive protein	Has a defence role in the body through clearance of pathogens and dead cells.	Inverse[25]
TNF-α	tumour necrosis factor-alpha	Regulates the immune system by reducing the infectious, immune, toxic, traumatic and ischaemic stimuli. Also improves inflammation through leukocyte adhesion, trans-endothelial migration and vascular leak.	Inverse[30]

hs-CRP, high sensitive-C-reactive protein; TNF-α, tumour necrosis Factor alpha; IL-12, interleukin 12; IFN-γ, interferon gamma; IL-6, interleukin 6; sICAM, soluble intercellular adhesion molecules; sVCAM, soluble vascular cell adhesion molecule; MPO, myeloperoxidase; HGF, hepatocyte growth factor

describes some commonly used biomarkers of both systemic and endothelial inflammation[21-30].

The active vitamin D hormone, $1,25(OH)2D_3$, can be produced in endothelial cells through activity of a specific endothelial α hydroxylase on circulating $25(OH)D_3$[31]. There is now an abundance of data that demonstrate the beneficial effects of $1,25(OH)2D_3$ on mediators of inflammation through the modulation of macrophage/monocytes and T and B lymphocytes. It also affects the differentiation of active CD4+ T-cells, enhances the inhibitory function of T-cells and promotes differentiation of monocyte into mature macrophages. Overall, a role in antibacterial and antiviral activities seems proven[32]. The logical extension of such observations would be that the correction of vitamin D deficiency or insufficiency must have

Table II. Vitamin D supplementation and endothelial function: summary of randomized controlled trials

Study reference number	Study location	Study details	Study quality score[1]	Vitamin D status achieved (nmol/l)	Study outcomes			Comments
					Endothelial function	Endothelial inflammation	Systemic inflammation	
35	Hong Kong	***Sample***: N= 100 T2DM patients ***Dose:*** 5000 IU D3/day or placebo **Duration:** 12 wk	5	Baseline: 52.75 ± 21.5 End: 86.8	No difference in FMD, or brachial-ankle PWV	No change in endothelial progenitor cells	No significant change in hs-CRP, oxidative stress markers	No differences in LDL-C, HDL-C or $HbA1_c$
36	USA	***Sample:*** N= 114 postmenopausal women, aged 60-70 yr ***Dose:*** 2500 IU/day D3 or placebo ***Duration:*** 16 wk	5	Baseline: 80 ± 26.3 End: + 39 ± 23.3	No difference in FMD, PWV or AIx		No significant difference in CRP	
37	USA	***Sample:*** N =57 African-American men and women, aged 19-50 yr ***Dose:*** 60,000 IU D3 per month (~2000 IU/d) or placebo ***Duration:*** 16 wk	4	Baseline: 34.3 ± 2.2 End: 100.9 ± 6.6	Significant improvement in FMD			No change in PTH, serum calcium or urinary calcium: creatinine ratio
38	USA	***Sample***: N= 90 CAD patients ***Dose:*** 50,000 IU D2 per week or placebo ***Duration***: 12 wk	4	Baseline: 85.75 ± 5.5 End; 100 ± 45	No difference in RH-PAT score	No difference in sICAM, sVCAM, or E-selectin	No difference in IL-12, IFN-γ, hs-CRP or IL-6	
39	Switzerland	***Sample***: N= 62 subjects with peripheral artery disease ***Dose:*** Single dose of 100,000 IU D3 or placebo ***Duration***: 4 wk	5	Baseline: 40.8 ± 16.8 End: 60.75 ± 15.5	No difference in endothelial function or arterial stiffness		No significant change in CRP, D-dimer, PAI-1	Short duration, low power
40	UK	***Sample:*** 50 South Asian women living in UK ***Dose***: 100,000 IU oral vitamin D3 or placebo ***Duration:*** 8 wk	5	Baseline: 27 ± 13 End: +16 by wk 4 and +10 nmol/l by wk 8	No difference in FMD	Platelet activation inhibitor-1 and tissue plasminogen activator levels fell significantly in the vitamin D group relative compared with placebo	No significant change in markers of inflammation	No significant change in insulin resistance

Contd..

Study reference number	Study location	Study details	Study quality score[1]	Vitamin D status achieved (nmol/l)	Study outcomes			Comments
					Endothelial Function	Endothelial inflammation	Systemic inflammation	
41	UK	***Sample:*** N=61 T2DM with <100 nmol/l status ***Dose:*** 3 groups: Single dose of 100,000 IU or 200,000 IU or placebo ***Duration:*** 16 wk	5	100,000 IU group: Baseline: 41 ± 14 End: 63 ± 20 200,000 IU group Baseline: 48 ± 21 End: 79 ± 31	No difference in FMD		Improvement in B type natriuretic peptide	BP was reduced in the vitamin D groups. Insulin resistance was similar
42	UK	***Sample:*** N= 75 patient with a history of myocardial infarction ***Dose:*** 100,000 IU of oral vitamin D3 (at baseline, 2 months and 4 months) or placebo ***Duration:*** Three doses at baseline, 2 months and 4 months	5	Baseline: 49 ± 20 End: +7 after 2 months; +13 after 6 months	No difference in endothelial function as measured by peripheral artery tonometry	No differences in TNF-α, E-selectin, or vWF	Significant reduction in CRP	
43	UK	***Sample:*** N= 58 patients with history of stroke and vitamin D <75 nmol/l ***Dose***: 100,000 IU oral vitamin D2 or placebo. ***Duration:*** 16 wk	4	Baseline: 38.7± (17.6) End: 54 ± 15 in 8 wk	Significant improvement in FMD at 8 wk but not 16 wk			No significant change in diastolic blood pressure
44	UK	***Sample:*** N= 159 aged > 70 yr & vitamin D level < 75 nmol/l) ***Dose:*** 100,000 IU D3/3 months over one year or placebo ***Duration:*** 52 wk	5	Baseline: 45 nmol/l End: +20	No difference in FMD, and arterial stiffness		No significant change in CRP or HOMA	No differences in cholesterol, glucose and blood pressure

T2DM, type two diabetes mellitus; FMD, follow mediated dilatation; hs-CRP, high sensitive-C-reactive protein; HOMA, homeostasis model assessment; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HbA1c, glycated haemoglobin; PWV, pulse wave velocity; IU, international unit; TNF-α, tumour necrosis factor alpha; CRP, C-reactive protein; IL-12, interleukin 12; IFN-γ, interferon gamma; IL-6, interleukin 6; Aix, augmentation index; RH-PAT score, reactive hyperemia peripheral arterial tonometry score; sICAM, soluble intercellular adhesion molecules: sVCAM, soluble vascular cell adhesion molecule; PTH, parathyroid hormone; vWF, Von Willebrand Factor; PAI-1, plasminogen activator inhibitor-1

some effect on endothelial function, possibly through abrogation of the inflammatory response, both systemic and endothelial. Recent outcomes from observational studies extend this point since hypovitaminosis D was directly associated with the extent of coronary artery disease determined by angiography[33].

The PubMed database was searched from 2009 to date. Key words used were vitamin D, supplementation, systemic inflammation, endothelium, endothelial dysfunction and humans. Selected articles were restricted to the English language and randomized controlled trials of vitamin D supplementation with some physical measures of endothelial function. All resultant studies were finally graded for their quality based on the score of Jadad *et al*[34] and only those 10 studies that met criteria of a good score (≥3) were included[35-44] (Table II).

It was perhaps surprising to find that the RCTs in this area did not support a role for the vitamin on endothelial function, with only two trials of eight showing an improvement. Moreover, of the many biomarkers of inflammation and endothelial activation reported, only three studies showed some improvement in either C-reactive protein, platelet activation inhibitor-1, tissue plasminogen activator and B type natriuretic peptide (Table II).

We restricted our search to one major database over the last five years. Perhaps a more extensive search strategy over a longer time frame was needed. While there were many methods for determining EF, the majority in this review used FMD which is regarded as the gold standard. Hence methodology may not be the issue here. The current value for adequate vitamin D status is 50 nmol/l and this is essentially meant to cover bone health. However, there are well argued views that even for bone health a value ≥ 75 nmol/l is essential[10,45,46]. It is possible that the target value may be much higher for non-skeletal endpoints. We have opined that the precise status achieved as well as the duration over which the target value is maintained, may be crucial to some extra-skeletal effects[7,47]. In the trials reviewed here (Table II), half the number had achieved a value between 85-100 nmol/l though one started from a baseline of 50 nmol/l and two from ~80 nmol/l. Duration of these trails was <16 wk, with only one lasting a year. It was not clear from these publications, for how long the achieved status had been maintained (Table II). These two facets may prove to be critical, as indicated by a RCT in South Asian women living in New Zealand. The authors of this study found a significant change in insulin resistance, only in those participants who achieved a value of 80 nmol/l at 12 wk and maintained that value until 24 wk[48]. While data like these are scarce, but provide the impetus for future trials to aim for specific 25(OH)D_3 levels and to maintain them over a defined period. Merely correcting vitamin inadequacy or deficiency may not be sufficient for extra-skeletal effects.

Conclusions

In this overview of vitamin D and endothelial function, it is found that the available evidence base does not support a role for the vitamin. Prospective studies could involve dose response trials that target a range of status values and maintain that target value for at least six months. In this regard, multicentre trials are a potential way forward to make such desirable outcomes applicable to the ethnic mix of their population, or across the world.

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References

1. Misra A, Chowbey P, Makkar B, Vikram N, Wasir J, Chadha D, *et al*. Consensus statement for diagnosis of obesity, abdominal obesity and the metabolic syndrome for Asian Indians and recommendations for physical activity, medical and surgical management. *J Assoc Physcians India* 2009; *57* : 163-70.
2. Harinarayan CV, Ramalakshmi T, Prasad UV, Sudhakar D, Srinivasarao PV, Sarma KV, *et al*. High prevalence of low dietary calcium, high phytate consumption, and vitamin D deficiency in healthy south Indians. *Am J Clin Nutr* 2007; *85* : 1062-7.
3. Sachan A, Gupta R, Das V, Agarwal A, Awasthi PK, Bhatia V. High prevalence of vitamin D deficiency among pregnant women and their newborns in northern India. *Am J Clin Nutr* 2005; *81* : 1060-4.
4. Goswami R, Marwaha RK, Gupta N, Tandon N, Sreenivas V, Tomar N, *et al*. Prevalence of vitamin D deficiency and its relationship with thyroid autoimmunity in Asian Indians: a community-based survey. *Br J Nutr* 2009; *102* : 382-6.
5. Nowson CA, McGrath JJ, Ebeling PR, Haikerwal A, Daly RM, Sanders KM, *et al*. Vitamin D and health in adults in Australia and New Zealand: a position statement. *Med J Aust* 2012; *196* : 686-7.
6. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, *et al*. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab* 2011; *96* : 53-8.

7. Soares MJ, Pathak K, Calton EK. Calcium and vitamin D in the regulation of energy balance: where do we stand? *Int J Mol Sci* 2014; *15* :4938-45.
8. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. *Am J Clin Nutr* 2008; *87* : 1080S-6S.
9. Peterlik M, Cross H. Vitamin D and calcium insufficiency-related chronic diseases: molecular and cellular pathophysiology. *Eur J Clin Nutr* 2009; *63* : 1377-86.
10. Heaney RP. Vitamin D in health and disease. *Clin J Am Soc Nephrol* 2008; *3* : 1535-41.
11. Szmitko PE, Wang C-H, Weisel RD, de Almeida JR, Anderson TJ, Verma S. New markers of inflammation and endothelial cell activation part I. *Circulation* 2003; *108* : 1917-23.
12. Sandoo A, van Zanten JJV, Metsios GS, Carroll D, Kitas GD. The endothelium and its role in regulating vascular tone. *Open Cardiovasc Med J* 2010; *4* : 302.
13. Johns DG, Charpie JR, Webb RC. Is ceramide signaling a target for vascular therapeutic intervention? *Curr Pharma Design* 1998; *4* : 481-8.
14. Pasimeni G, Ribaudo MC, Capoccia D, Rossi F, Bertone C, Leonetti F, *et al*. Non-invasive evaluation of endothelial dysfunction in uncomplicated obesity: Relationship with insulin resistance. *Microvasc Res* 2006; *71* : 115-20.
15. Tousoulis D, Antoniades C, Stefanadis C. Evaluating endothelial function in humans: a guide to invasive and non-invasive techniques. *Heart* 2005; *91* : 553-8.
16. Smith S, Greenland P, Grundy S. American Heart Association Conference Proceedings, Prevention Conference V: Beyond secondary prevention: identifying the high-risk patient for primary prevention. Executive summary. *Circulation* 2000; *10* : E12-6.
17. Barac A, Campia U, Panza JA. Methods for evaluating endothelial function in humans. *Hypertenion* 2007; *49* : 748-60.
18. Woodman R, Watts G. Measurement and application of arterial stiffness in clinical research: focus on new methodologies and diabetes mellitus. *Med Sci Monitor* 2003; *9* : RA81-9.
19. Soares MJ, Kuriyan R, Kurpad AV. Calcium and vitamin D modulate postprandial vascular function: A pilot dose–response study. *Diab Met Syn: Clin Res Rev* 2010; *4* : 128-31.
20. Huang AL, Vita JA. Effects of systemic inflammation on endothelium-dependent vasodilation. *Trends Cardiovas Med* 2006; *16* : 15-20.
21. Barber M, Langhorne P, Rumley A, Lowe GD, Stott DJ. Hemostatic function and progressing ischemic stroke D-dimer predicts early clinical progression. *Stroke* 2004; *35* : 1421-5.
22. Meigs JB, Hu FB, Rifai N, Manson JE. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. *JAMA* 2004; *291* : 1978-86.
23. Zhang R, Brennan M-L, Fu X, Aviles RJ, Pearce GL, Penn MS, *et al*. Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA* 2001; *286* : 2136-42.
24. Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. *Circulation* 2001; *103* : 491-5.
25. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 2001; *286* : 327-34.
26. Reinhart K, Bayer O, Brunkhorst F, Meisner M. Markers of endothelial damage in organ dysfunction and sepsis. *Crit Care Med* 2002; *30* : S302-S12.
27. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 1999; *17* : 189-220.
28. Ren J. Leptin and hyperleptinemia-from friend to foe for cardiovascular function. *J Endocrinol* 2004; *181* : 1-10.
29. Yasuda S, Goto Y, Baba T, Satoh T, Sumida H, Miyazaki S, *et al*. Enhanced secretion of cardiac hepatocyte growth factor from an infarct region is associated with less severe ventricular enlargement and improved cardiac function. *J Am Coll Cardiol* 2000; *36* : 115-21.
30. Zhang H, Park Y, Wu J, Chen X, Lee S, Yang J, *et al*. Role of TNF-alpha in vascular dysfunction. *Clin Sci* 2009; *116* : 219-30.
31. Zehnder D, Bland R, Chana RS, Wheeler DC, Howie AJ, Williams MC, *et al*. Synthesis of 1, 25-dihydroxyvitamin D3 by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. *J Am Soc Nephr* 2002; *13* : 621-9.
32. Lai Y-H, Fang T-C. The pleiotropic effect of Vitamin D. *ISRN Nephr* 2013; Article ID898125 : 6 pages. Available from: *http://dx.doi.org/10.5402/2013/898125*.
33. Verdoia M, Schaffer A, Sartori C, Barbieri L, Cassetti E, Marino P, *et al*. Vitamin D deficiency is independently associated with the extent of coronary artery disease. *Eur J Clin Invest* 2014; *44* : 634-42.
34. Jadad AR, Moore RA, Carroll D, Jenkinson C, Reynolds DJM, Gavaghan DJ, *et al*. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Contr Clin Trials* 1996; *17* : 1-12.
35. Yiu YF, Yiu KH, Siu CW, Chan YH, Li SW, Wong LY, *et al*. Randomized controlled trial of vitamin D supplement on endothelial function in patients with type 2 diabetes. *Atherosclerosis* 2013; *227* : 140-6.
36. Gepner AD, Ramamurthy R, Krueger DC, Korcarz CE, Binkley N, Stein JH. A prospective randomized controlled trial of the effects of vitamin D supplementation on cardiovascular disease risk. *PLoS One* 2012; *7* : e36617.
37. Harris RA, Pedersen-White J, Guo DH, Stallmann-Jorgensen IS, Keeton D, Huang Y, et al. Vitamin D3 supplementation for 16 weeks improves flow-mediated dilation in overweight African-American adults. *Am J Hypertens* 2011; *24* : 557-62.
38. Sokol SI, Srinivas V, Crandall JP, Kim M, Tellides G, Lebastchi AH, *et al*. The effects of vitamin D repletion on endothelial function and inflammation in patients with coronary artery disease. *Vasc Med* 2012; *17* : 394-404.
39. Stricker H, Tosi Bianda F, Guidicelli-Nicolosi S, Limoni C, Colucci G. Effect of a single, oral, high-dose vitamin D supplementation on endothelial function in patients with peripheral arterial disease: a randomised controlled pilot study. *Eur J Vasc Endovasc Surg* 2012; *44* : 307-12.

40. Witham MD, Adams F, Kabir G, Kennedy G, Belch JJ, Khan F. Effect of short-term vitamin D supplementation on markers of vascular health in South Asian women living in the UK--a randomised controlled trial. *Atherosclerosis* 2013; *230* : 293-9.

41. Witham MD, Dove FJ, Dryburgh M, Sugden JA, Morris AD, Struthers AD. The effect of different doses of vitamin D(3) on markers of vascular health in patients with type 2 diabetes: a randomised controlled trial. *Diabetologia* 2010; *53* : 2112-9.

42. Witham MD, Dove FJ, Khan F, Lang CC, Belch JJ, Struthers AD. Effects of vitamin D supplementation on markers of vascular function after myocardial infarction--a randomised controlled trial. *Int J Cardiol* 2013; *167* : 745-9.

43. Witham MD, Dove FJ, Sugden JA, Doney AS, Struthers AD. The effect of vitamin D replacement on markers of vascular health in stroke patients - a randomised controlled trial. *Nutr Metab Cardiovasc Dis* 2012; *22* : 864-70.

44. Witham MD, Price RJ, Struthers AD, Donnan PT, Messow CM, Ford I, *et al*. Cholecalciferol treatment to reduce blood pressure in older patients with isolated systolic hypertension: the VitDISH randomized controlled trial. *JAMA Intern Med* 2013; *173* : 1672-9.

45. Dawson-Hughes B, Heaney RP, Holick MF, Lips P, Meunier PJ, Vieth R. Estimates of optimal vitamin D status. *Osteoporos Int* 2005; *16* : 713-6.

46. Vieth R. What is the optimal vitamin D status for health? *Prog Biophys Mol Bio* 2006; *92* : 26-32.

47. Pathak K, Soares M, Calton E, Zhao Y, Hallett J. Vitamin D supplementation and body weight status: a systematic review and meta-analysis of randomized controlled trials. *Obes Rev* 2014; *15* : 528-37.

48. von Hurst PR, Stonehouse W, Coad J. Vitamin D supplementation reduces insulin resistance in South Asian women living in New Zealand who are insulin resistant and vitamin D deficient-a randomised, placebo-controlled trial. *Br J Nutr* 2010; *103* : 549-55.

Reprint requests: Dr Mario J. Soares, Associate Professor, Directorate of Nutrition Dietetics & Food Technology, School of Public Health, Curtin University, Bentley Campus, Kent Street, Perth Western Australia 6845, Australia
e-mail: m.soares@curtin.edu.au

To Whom It May Concern

I, **Ali Mahdi Alyami**, contributed *(***designed the table and wrote the first draft***)* to the paper/publication entitled *(***Alyami, A., Soares, M. J., Sherriff, J. L., & Mamo, J. C. (2014). Vitamin D & endothelial function.** ***The Indian journal of medical research****, 140*(4), 483.*)*.

Candidate: Ali Alyami **Signature:** _____

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Co-Author 1: Mario Soares **Signature:** _____

Co-Author 2: Jillian Sherriff **Signature:** _____

Co-Author 3: John Mamo: **Signature:** _____

Overall Objectives

The overall objectives of this PhD research were to examine the associations between vitamin D status and endothelial function, and the role of insulin resistance and systemic inflammation in this context.

To address these objectives, we planned the following studies to test our hypotheses.

Hypothesis & Studies Conducted

Hypothesis 1:

Cholecalciferol supplementation will improve endothelial function and reduce systemic inflammation.

Study 1: Development and execution of a systematic review and meta-analysis protocol examining the effect of cholecalciferol supplementation on endothelial function and systemic inflammatory biomarkers.

Hypothesis 2:

Low circulating 25(OH)D status will be associated with dyslipidaemia and endothelial dysfunction as gauged from endothelial cell activation molecules.

Study 2: A cross sectional study comparison of circulating lipids and endothelial cell activation molecules between tertiles of vitamin D status.

Hypothesis 3:

Ethnic differences in low circulating 25(OH)D status will account for endothelial dysfunction following glucose ingestion.

Study 3: A cross sectional study comparison of Middle Eastern and Australian men of European descent, following glucose-induced endothelial changes measured by pulse contour analysis (Pulse Trace 2000).

Chapter 2:

A systematic review of high quality randomised controlled trials examining the effect of cholecalciferol supplementation on endothelial function.

Chapter 2 Part 1:

Paper: A systematic review protocol examining the effect of vitamin D supplementation on endothelial function

Alyami, A., Soares, M. J., Sherriff, J. L., Zhao, Y., Hallett, J., & Coombes, F. (2015). A systematic review protocol examining the effect of vitamin D supplementation on endothelial function. BMJ open, 5(6), e006835. [Impact factor =2.562; cited by 1]. Please see the original paper next page. (Co-author permission signatures attached)

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A systematic review protocol examining the effect of vitamin D supplementation on endothelial function

A Alyami,[1] MJ Soares,[1] JL Sherriff,[1] Y Zhao,[2] J Hallett,[3] F Coombes[4]

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[1]Directorate of Nutrition, Dietetics & Food Technology, School of Public Health, Curtin Health Innovation Research Institute of Ageing & Chronic Disease, Curtin University, Perth, Western Australia, Australia
[2]Directorate of Epidemiology & Biostatistics, School of Public Health, Curtin University, Perth, Western Australia, Australia
[3]Collaboration for Evidence, Research and Impact in Public Health, School of Public Health, Curtin University, Perth, Western Australia, Australia
[4]University Health Service, Support Services, Curtin University, Perth, Western Australia, Australia

Correspondence to
Dr MJ Soares;
m.soares@curtin.edu.au

ABSTRACT

Introduction: Vitamin D has potential benefits for extraskeletal health. These could include an anti-inflammatory effect as well as a reduction in endothelial dysfunction. We aim to provide quality evidence for the hypothesis that supplementation with vitamin D will improve endothelial function (EF), possibly through the abrogation of systemic inflammation.

Methods and analysis: We will conduct a systematic review of all randomised controlled trials on vitamin D supplementation and EF lasting 12 weeks or more. The search will cover the period 2000–2015 and include studies that describe direct measures of EF, markers of endothelial cell (EC) activation and if concurrently reported, indicators of systemic inflammation. Study selection will follow the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and study quality will be assessed by the Jadad score in addition to an evaluation of allocation concealment and data analysis. If sufficient data are available, a meta-analysis will be conducted. The effect sizes will be generated using Hedges' g score, for both fixed and random effect models. I^2 statistics and Galbraith plots will be used to assess heterogeneity and identify their potential sources. Potential publication and small sample size bias will be assessed by visual inspections of funnel plots and also Egger's test. Meta-regression analysis (if feasible) will be conducted with restricted maximum likelihood (REML) estimation method, controlling for potential confounders (demographics, study methods, location, etc). A backward elimination process will be applied in the regression modelling procedure. Subgroup analysis, conditional on number of studies retrieved and their sample size, will be stratified on participant disease category, total dose administered, degree of 25(OH)D change and type of supplement used.

Ethics and dissemination: Formal ethical approval is not required as primary data will not be collected. The results will be disseminated through a peer-reviewed publication, conference presentation and the popular press.

Trial registration number: International Prospective Register for Systematic Reviews (PROSPERO) number CRD42014013523.

Strengths and limitations of this study

- Systematic review and meta-analysis of randomised controlled trials.
- Will offer highest level of evidence for informed decisions.
- Potential clarification that the effect of vitamin D status on endothelial dysfunction is through inflammation.
- Availability of quality studies with direct measures of endothelial function based on the same technique.
- The many inflammatory and endothelial activation biomarkers in the literature hamper collation of outcomes.
- Key words: endothelial function, vitamin D, inflammation, systematic review, randomised controlled trials.

INTRODUCTION

The vascular endothelium plays a pivotal role in the detection and response to blood-borne signals and changes in haemodynamic forces. Endothelial dysfunction is strongly linked to cardiovascular disease (CVD),[1] and can predict the occurrence of type 2 diabetes (T2DM).[2] A chronic low-grade inflammation is common to many metabolic disorders,[3] and also underscores endothelial dysfunction.[4 5] Vitamin D inadequacy is now a global issue and normalisation of status has a potential protective role in conditions such as obesity, CVD and T2DM.[6–11] Vitamin D status is related to inflammation.[12 13] The conversion of 25(OH)D to its active form 1,25(OH)2D occurs in immune system cells such as dendritic cells, macrophages, T cells and B cells.[14] The outcome of 1,25(OH)2D action in these cells is a decreased production of interferon γ (INF-γ), interleukin-12 (IL-12), interleukin-6 (IL-6), and interleukin-23(IL-23) with an enhanced production of IL-4.[14] Vitamin D may also benefit endothelial function (EF).[15 16] The endothelium can convert 25(OH)D to its active form through a specific endothelial 1 α-hydroxylase.[17] Interestingly, greater enzyme activity is stimulated by inflammatory cytokines.[18] Protective effects of vitamin D may then be realised through

increased nitric oxide (NO) production, decreased oxidative stress, reduced IL-6, vascular cell adhesion molecules (VCAM) and intracellular adhesion molecule (ICAM) among other effects.[16] Thus the overall impact of adequate vitamin D in this context would be to decrease both systemic inflammation and endothelial dysfunction.

One of the most important actions of endothelium is the production of NO, which plays a major role in regulating the vessel diameter and, hence resistance, throughout the arterial bed.[19] However, two other endothelial cell (EC) products, endothelium-derived hyperpolarising factor and prostacyclin (PGI2), also produce vasodilation of the underlying smooth muscle cells, and overall these actions are countered by the vasoconstrictor EC factor endothelin-1 in the regulation of vascular tone.[15] Endothelial dysfunction is characterised by reduced NO-dependent vascular activity which leads to dysregulation of arterial tone.[20] However, there is an endothelium independent pathway as well that is determined by the activity of the smooth muscle layer. Sublingual glyceryl trinitrate (GTN) can be used to uncover the influence of this pathway,[21] as GTN decreases smooth muscle tone leading to vasodilation.[21] A comprehensive assessment of EF usually encompasses the testing of both pathways.

EF is assessed by testing the vascular reactivity of either coronary or peripheral arteries. Initially the invasive technique of artery catheterisation was used; this assesses endothelial-dependent vasodilation. Subsequently, non-invasive techniques like flow-mediated dilation (FMD) were developed. This is the current gold standard for measuring EF in peripheral arteries. FMD uses ultrasound imaging to detect the endothelial response to shear stress, acetylcholine infusion, and salbutamol inhalation for the assessment of the endothelial-dependent pathway, or sublingual GTN for the endothelial-independent pathway. More recently, pulse contour analysis (PCA), based on a photoplethysmographic recording of the digital volume pulse, has been used to assess EF.[22] The derived variables, stiffness index and reflective index (RI) reflect large artery stiffness and small artery vascular tone, respectively.[23] Studies that employ both salbutamol and GTN in conjunction with PCA have been used to report endothelial dysfunction. On the other hand, arterial applanation tonometry uses a sensitive probe applied in turn to the carotid and femoral arteries to detect characteristics of the transmitted waveform. A derived variable is the augmentation index (AIx), the ratio of the pulse pressure at the second systolic peak to the pulse pressure of the first systolic peak.[24] Other studies have also employed markers of EC activation, like higher plasma levels of soluble VCAM, ICAM, P-selectin and E-selectin as indicators of endothelial dysfunction.[15] Systemic inflammation is usually measured by the levels of circulating inflammatory biomarkers such as high sensitivity C reactive protein (CRP), white cell count (WCC), INF- γ, IL-12, IL-6, IL-23 and IL-4.[14 25] CRP is an interesting marker since its effect on EC activation[5] could underscore its high prediction of CVD.[5 25] Moreover, strong relationships between WCC, ICAM and fibrinogen and the prediction of CVD has also been documented.[1 26]

Collectively, there is sufficient evidence to hypothesise that adequate vitamin D status may directly attenuate endothelial dysfunction (as supported from functional measures and/or markers of EC activation), or act indirectly through the abrogation of systemic inflammation. Two limited narrative reviews on randomised controlled trials (RCTs) did not, however, uncover consistent support for an effect of vitamin D on improvements in EF or decreases in markers of EC activation.[15 16] Clearly there is a need to expand the scope of such findings to arrive at an evidence-based conclusion. To our knowledge there is no published systematic review that addresses our question. Previous systematic reviews in related fields have examined the links between vitamin D and CRP,[27] and vitamin D and blood pressure,[28] while a narrative review reported on vitamin D, blood pressure, endothelial and renal function of postmenopausal women.[29] The present systematic review protocol will evaluate potential causal interrelationships between vitamin D status and EF, and determine whether systemic inflammation is a moderator of the effect. We address our objectives through a comprehensive protocol targeting all RCTs in this area, from 2000 to 2015, in order to confirm or negate this extraskeletal role for vitamin D.

METHODS AND DESIGN

Population

The systematic review will include high quality RCTs on adults aged >20 years who have been supplemented with cholecalciferol or calcitriol, and have had measures of EF, EC activation and/or systemic inflammation before and after the interventions. The study population will be restricted to healthy subjects, and overweight or obese who suffer from glucose intolerance, CVD, metabolic syndrome (MetS) or T2DM.

Study design

This systematic review will consider only randomised controlled trials of good quality.

Search strategy

The search strategy aims to find published articles only, and will include a three-stage protocol (figure 1). An initial limited search of Medline and Scopus will be undertaken; this will be followed by analysis of the text words contained in the titles and abstracts, and of the index terms used to describe each article. A second search, using all identified keywords and index terms, will then be undertaken across all included databases. In the third step, the reference lists of key articles will be searched for additional studies. Studies will be restricted to the English language and to those published from 2000 to 2015, inclusive. The databases that will be

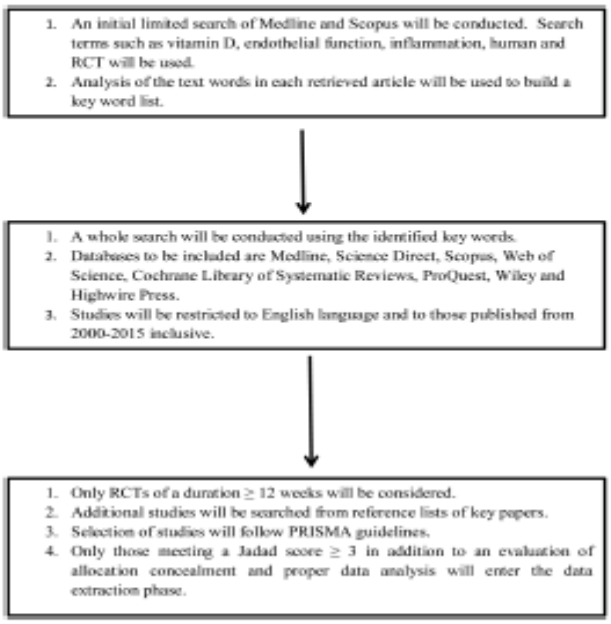

Figure 1 A schematic of the proceses of the systemic review. (Randomised controlled trials (RCTs); Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)).

searched are Medline, Science Direct, Scopus, Web of Science, Cochrane Library of Systematic Reviews, ProQuest, Wiley and Highwire Press.

Study selection

Quantitative studies will be independently assessed by three reviewers and reported using the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram.[50] Valid studies will then be assessed for their quality before any retrieval of information. Any disagreements that arise between the reviewers will be resolved through discussion.

Quality assessment

The three reviewers will independently check each selected article to minimise bias. All selected articles will be judged for their quality based on the Jadad score[51] in addition to an evaluation of allocation concealment and data analysis.[52]

Data extraction

Quantitative data will be extracted from papers receiving a Jadad score of 3 and over with adequate allocation concealment and proper data analysis.[51 52] The data extracted will include all details specific to the review question and fulfils the requirements for both the narrative synthesis of outcomes and the potential meta-analysis. We will also contact corresponding authors for key information when data are ambiguous or missing from the published study. Data extraction will be independently cross-checked.

Outcomes

The outcomes of the review will be grouped under the following headings

- EF: this will include direct measures as measured by flow mediated dilation (FMD), PCA, AIx or endothelial vasodilation/vasoconstriction following drug intervention.
- EC activation: these include circulating markers such as P-selectin, E-selectin, L-selectin, VCAM-1, ICAM-1 or von Willebrand factor.
- Systemic inflammatory molecules: these will include markers, such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-KB), pro-inflammatory cytokine IL-6, IL-12, and high-sensitivity CRP, as well as anti-inflammatory cytokines such as IL-6, IL-10, and adiponectin.

ANALYSIS

Descriptive analysis

A narrative synthesis of the outcomes of the selected studies will be presented in the final review. This will include the following:

1. Type of intervention and the control group and sample size;
2. 25(OH)D3 baseline and final measurement and other biomarkers of interest;
3. Targeted population and its characteristics; age, sex, ethnicity, disease prevalence in group, location and the distance from equator, if possible;
4. Intervention outcomes: this will include the change in 25(OH)D, measurements of EF such as FMD, AIx and PCA derived end points RI and stiffness index, systemic inflammatory biomarkers, EC activation biomarkers, time between last dose and EF measurement.

Statistical analysis

We are interested in the relationship between vitamin D supplementation and endothelial dysfunction. Endothelial dysfunction is measured in different ways among studies; therefore, we anticipate a limited ability to run a meta-analysis for this review. However, in studies which used the same end point measurements we will report pre-intervention, postintervention and overall mean change pertaining to the endothelial dysfunction outcomes of interest. The overall mean change will be calculated by subtracting the mean change in the placebo group from that in the treatment group in the studies if these had a parallel design. Standard deviation (SD) will be calculated from standard errors (SEs), or confidence interval (95% CI), or t or F value from raw data, where available, for both the placebo group and the treatment group for each study included.

Meta-analysis (where possible) will be carried out to assess the effect of vitamin D supplementation on

measures of EF and systemic/vascular inflammation. Effectiveness of vitamin D supplementation on endothelial dysfunction will be reported as standardised mean difference (SMD) for each individual study and its 95% CI. A positive SMD will denote a higher (more favourable) value in the vitamin D3 group. The effect sizes will be generated using Hedges' g score and presented using a forest plot for each study to assess the magnitude of the intervention effect on a particular outcome. The overall effect sizes will be estimated using both fixed-effects models and random-effects models. I^2 statistics and Galbraith plot will be used to assess for heterogeneity and identify the potential sources of heterogeneity. Subgroup analyses, conditional on number of studies retrieved and their sample size, will be stratified on participant disease category (eg, CVD/MetS/T2DM) or total dose administered (daily dose × duration) (low-medium-high) or degree of 25(OH)D change (low-medium-high) or supplement used (calcitriol vs vitamin D3 alone vs calcium + vitamin D3).

Potential publication and small sample size bias will be assessed by visual inspections of funnel plots and also Egger's test. To explore the effect of main factors of interest on predicting SMD, meta-regression analysis will be conducted with restricted maximum likelihood (REML) estimation method, controlling for potential confounders (demographics, study methods, and location). Backward elimination process will be applied in the regression modelling procedure. All of the statistical analysis will be performed by using STATA V.12.0 (StataCorp, College Station, Texas, USA).[55] A p value<0.05 will be considered statistically significant for all analyses.

CONCLUSION

This systematic review will provide evidence in support or against the hypothesis that vitamin D has a role in EF. This conclusion will stem from direct measurements of EF and/or EC activation, and indirectly through changes in biomarkers of systemic inflammation. Where sufficient data are available, we will conduct a meta-analysis to confirm the relationship between the improvement in vitamin D status and the reduction in endothelial dysfunction. Moreover, whether this occurs through a reduction in systemic inflammation will also be clarified. Overall, the review will complement the evidence base on the extraskeletal benefits of vitamin D.

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Contributors AA, MJS, JLS and JH conceived the idea, planned and designed the study protocol. AA designed the figure and wrote the first draft; YZ planned the data extraction and statistical analysis; FC provided critical insights. All authors have approved and contributed to the final written manuscript.

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REFERENCES

1. Pearson TA, Mensah GA, Alexander RW, *et al*. Markers of inflammation and cardiovascular disease application to clinical and public health practice: a statement for healthcare professionals from the centers for disease control and prevention and the American Heart Association. *Circulation* 2003;107:499–511.
2. Meigs JB, Hu FB, Rifai N, *et al*. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. *JAMA* 2004;291:1978–86.
3. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006;444:860–7.
4. Huang AL, Vita JA. Effects of systemic inflammation on endothelium-dependent vasodilation. *Trends Cardiovasc Med* 2006;16:15–20.
5. Szmitko PE, Wang CH, Weisel RD, *et al*. New markers of inflammation and endothelial cell activation part I. *Circulation* 2003;108:1917–23.
6. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. *Amer J Clin Nutr* 2008;87:1080S–6S.
7. Peterlik M, Cross H. Vitamin D and calcium insufficiency-related chronic diseases: molecular and cellular pathophysiology. *Euro J Clin Nutr* 2009;63:1377–86.
8. Heaney RP. Vitamin D in health and disease. *Clin J Am Soc Nephrol* 2008;3:1535–41.
9. Hossein-nezhad A, Holick MF. Vitamin D for health: a global perspective. *Mayo Clin Proc* 2013;88:720–55.
10. Mitri J, Muraru M, Pittas A. Vitamin D and type 2 diabetes: a systematic review. *Eur J Clin Nutr* 2011;65:1005–15.
11. Soares MJ, Pathak K, Calton EK. Calcium and vitamin d in the regulation of energy balance: where do we stand? *Int J Mol Sci* 2014;15:4938–45.
12. Lai YH, Fang TC. The pleiotropic effect of Vitamin D. *ISRN Nephrol* 2013;2013:898125.
13. Calton E, Keane K, Soares M. The potential regulatory role of vitamin D in the bioenergetics of inflammation. *Cur Opin Clin Nutr Metab Care* 2015;18:367–73.
14. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol* 2008;8:685–98.
15. Alyami A, Soares M, Sherriff J, *et al*. Vitamin D & endothelial function. *Indian J Med Res* 2014;140:483–90.
16. Dalan R, Liew H, Tan WKA, *et al*. Vitamin D and the endothelium: basic, translational and clinical research updates. *IJC Metab Endocr* 2014;4:4–17.
17. Merke J, Milde P, Lewicka S, *et al*. Identification and regulation of 1, 25-dihydroxyvitamin D3 receptor activity and biosynthesis of 1, 25-dihydroxyvitamin D3. Studies in cultured bovine aortic endothelial cells and human dermal capillaries. *J Clin Invest* 1989;83:1903–15.
18. Zehnder D, Bland R, Chana RS, *et al*. Synthesis of 1, 25-dihydroxyvitamin D3 by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. *J Am Soc Nephrol* 2002;13:621–9.
19. Goodfellow J, Owens D, Henderson A. Cardiovascular syndromes X, endothelial dysfunction and insulin resistance. *Diabetes Res Clin Pract* 1996;31:S163–71.
20. Kim JA, Montagnani M, Koh KK, *et al*. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation* 2006;113:1888–904.
21. Yaginuma T, Avolio A, O'Rourke M, *et al*. Effect of glyceryl trinitrate on peripheral arteries alters left ventricular hydraulic load in man. *Cardiovasc Res* 1986;20:153–60.
22. Woodman RJ, Watts GF. Measurement and application of arterial stiffness in clinical research: focus on new methodologies and diabetes mellitus. *Med Sci Monit* 2003;9:RA81–9.
23. Woodman RJ, Watts GF, Kingwell BA, *et al*. Interpretation of the digital volume pulse; its relationship with large and small artery compliance. *Clin Sci* 2003;104:283–5.

24. Barac A, Campia U, Panza JA. Methods for evaluating endothelial function in humans. *Hypertension* 2007;49:748–60.
25. Ridker PM. Inflammatory biomarkers and risks of myocardial infarction, stroke, diabetes, and total mortality: implications for longevity. *Nutr Rev* 2007;65(Suppl 3):S253–9.
26. Witkowska AM. Soluble ICAM-1: a marker of vascular inflammation and lifestyle. *Cytokine* 2005;31:127–34.
27. Chen N, Wan Z, Han SF, *et al*. Effect of vitamin D supplementation on the level of circulating high-sensitivity C-reactive protein: a meta-analysis of randomized controlled trials. *Nutrients* 2014;6:2206–16.
28. Beveridge LA, Struthers AD, Khan F, *et al*. Effect of vitamin D supplementation on blood pressure: a systematic review and meta-analysis incorporating individual patient data. *JAMA Intern Med* 2015;175:745–54.
29. Liu ZM, Woo J, Wu SH, *et al*. The role of vitamin D in blood pressure, endothelial and renal function in postmenopausal women. *Nutrients* 2013;5:2590–610.
30. Moher D, Liberati A, Tetzlaff J, *et al*. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Ann Intern Med* 2009;151:264–9.
31. Jadad AR, Moore RA, Carroll D, *et al*. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Control Clin Trials* 1996;17:1–12.
32. Centre for Evidence in Transplantation. Assessment of randomised controlled trials (RCTs). 2010. http://www.transplantevidence.com/library_rct.php (accessed 08–08–2014).
33. Hamilton L. Statistics with STATA: Version 12. USA: Cengage Learning, 2012.

To Whom It May Concern

I, **Ali Mahdi Alyami**, contributed *(***designed the figure and wrote the first draft***)* to the paper/publication entitled **(Alyami, A., Soares, M. J., Sherriff, J. L., Zhao, Y., Hallett, J., & Coombes, F. (2015). A systematic review protocol examining the effect of vitamin D supplementation on endothelial function. BMJ open, 5(6), e006835.)**

Candidate: Ali Alyami **Signature:** _____

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Co-Author 1: Mario Soares **Signature:** _____

Co-Author 2: Jillian Sherriff **Signature:** _____

Co-Author 3: Yun Zhao **Signature:** _____

Co-Author 4: Jonathan Hallett **Signature:** _____

Co-Author 5: Fiona Coombes **Signature:** _____

Chapter 2 part 2:

A systematic review of high quality randomised controlled trials examining the effect of cholecalciferol supplementation on endothelial function.

Introduction

The role of the endothelium is to maintain vascular integrity and hence haemodynamic physiology (26). Poor vascular health plays a significant role in the initiation and progression of cardiovascular (CVD) and metabolic disease (193). Chronic disease progression such as atherosclerotic change in CVD and insulin resistance (IR) in type 2 diabetes mellitus (T2DM), has been linked to varying degrees of endothelial dysfunction (ED) (194-197). Studies show that impaired endothelial function (EF) is one of the first pathological events during atherosclerotic change (198, 199).

ED is a multifaceted process involving several pathophysiological mechanisms including imbalance of vasoactive molecules, pro-inflammatory cytokine production, liberation of reactive oxygen species (ROS), and the generation of pro-atherogenic lipoproteins (199, 200). The reduction of endothelium-derived nitric oxide (NO) in ED potentiates the inflammatory process via limiting platelet and leucocyte adhesion and vascular smooth muscle cell proliferation (199-201). Vitamin D is an important pro-hormone recognised predominantly for its role in bone remodelling and Ca^{2+} homeostasis (59). Nonetheless, recent evidence has suggested numerous extra-skeletal roles of extracellular vitamin D (69). Indeed, vitamin D receptors (VDRs) are ubiquitously expressed throughout the body, including in endothelial cells, vascular smooth muscle and cardiac cells (33).

The active form of vitamin D, calcitriol [$1,25(OH)_2D$], has been implicated in vascular endothelial function, primarily through its potential attenuation of the pro-inflammatory process (34). Calcitriol has been shown to regulate vascular smooth muscle tone, in addition to regulating both the relationship between insulin and glucose, via possible role in improving insulin sensitivity and secretion, and the immune functionality of endothelial cells (34, 202). The former action is exerted through the transcriptional regulation of endothelial nitric oxide synthase (eNOS) (168, 169). This relationship has been demonstrated in animal models, where VDR knockout in mice demonstrates that low calcitriol levels cause endothelial dysfunction which culminates in poor arterial smooth muscle cell flexibility and cardiac dysfunction (203).

Notwithstanding these findings, observational analyses examining the association between circulating 25(OH)D and EF have produced mixed results in terms of cardiovascular and metabolic risks. A large body of evidence supports an inverse relationship between low plasma 25(OH)D and high cardiovascular risk, including chronic disease progression such as myocardial infarction (MI), cerebrovascular accidents, hypertension and T2DM (204-206). Our own data indicate a role for both dietary calcium and plasma 25(OH)D in regulating vascular tone of younger adults (207) as well as in preventing dyslipidaemia and in endothelial cell activation (208). Such outcomes are not always observed in other study populations (209, 210). Differences could be ascribed to variance in study design, such as hypovitaminosis D cut-off values for study entry, follow-up duration, criteria applied to the diagnosis of adverse health events, and a variety of confounding factors such as diet, exercise, sun expo, and seasonality; or characteristics and health status of the populations assessed. RCTs evaluating the effect of vitamin D supplementation on EF have depicted similarly conflicting outcomes; several studies have demonstrated improvements in EF (171, 211, 212), while other authors report non-significant changes (172-183). However, many of these RCTs were excluded from our systematic review because of the inclusion of unhealthy study participants, which may have influenced the trial outcomes.

Previous studies linking vitamin D supplementation, with or without calcium, produced different results. In 2010, a systematic review examining studies published from 1966 to 2009 found that vitamin D supplementation reduces CVD risks, whereas calcium supplementation has minimal impact on CVD risks (213). Similarly, a 2016 systematic review of studies from 1966 to 2016, examining the possible link between calcium intake and CVD risk, found no effect of calcium intake of 2000-2500 mg/day on CVD risk in healthy adults (214). Another RCT among postmenopausal women found an association between calcium supplementation (200 mg/day) and myocardial infarction and stroke (215). There is some evidence for vitamin D supplementation and little evidence for a

beneficial effect of calcium with some evidence that calcium has adverse effect on cardiovascular health.

A 2007 systematic review demonstrated that vitamin D supplementation, with or without calcium, has a beneficial effect on glucose metabolism (216). Another randomised control trial (RCT) found that vitamin D supplementation improves β cell function, whereas calcium supplementation had no effect (217). Another RCT conducted on non-diabetic adults over three years found that daily vitamin D supplementation with calcium, reduces high blood sugar and insulin resistance with time, for those with impermanent fasting glucose (IFG) (218). A large cohort study conducted over 20 years on women found they were less likely to develop T2DM if there was supplementation with >1200 mg/day of calcium (21% less likely), > 800 IU/day of vitamin D (13% less likely) or both (33% less likely) (205). A RCT found no beneficial effect of supplementation with vitamin D (400 IU/day) and calcium (1000 mg/day) for 2,291 newly diagnosed diabetic women in relation to diabetes risk or glucose metabolism (219). An Australian cohort study found that those who did not develop diabetes had a higher calcium intake (923 mg/day) compared to those who developed diabetes (881 mg/day) (220). An RCT conducted over 12 weeks revealed a beneficial effect on blood glucose levels in diabetic participants with the consumption of vitamin D fortified yoghurt, with or without calcium, compared to the placebo group (221). In conclusion, there are some supporting evidences for the role of vitamin D and calcium on the prevention of T2DM.

Objective measurement of EF can be conducted utilising several methods based around the assessment of vascular reactivity, either in the coronary or peripheral arteries. Indeed, the methodological approach differs based on resources, technical experience and the hypothesis being investigated. Invasive arterial catheterisation was originally used in order to measure endothelial-dependent vasodilation (53). However this method was invasive, thus non-invasive contemporary methods are more practicable. Non-invasive measurement of the peripheral arteries, such as the brachial artery, is typically achieved using flow-mediated dilation (FMD), however this method requires a high level of

expertise and thus alternative methods requiring less expertise were developed (53, 54). These newer methods include pulse wave velocity (PWV), augmentation index (AIx), reactive hyperemia index using EndoPAT (RHI-PAT) and pulse contour analysis (PCA) (47, 53, 54, 222, 223). Also, these new methods include the measurement of systemic inflammation biomarkers such as C-reactive protein (CRP), interleukin 6 (IL-6), tumour necrosis factor alpha (TNFα) and/or endothelial cell activation molecules for example, soluble vascular cellular adhesion molecule sVCAM, soluble intracellular adhesion molecule sICAM, P-selectin and E-selectin (26, 47).

Vitamin D inadequacy is highly prevalent globally and evidence suggests that improvement in status may be linked to improvement in endothelial function (5). A systematic review of RCTs was conducted to explore the evidence for a causative relationship between poor vitamin D status and ED.

Methods

Search Strategy

We conducted a systematic review of all RCTs that evaluated cholecalciferol supplementation and EF and that had durations of ≥12 weeks and assessed participants aged ≥18 years. The systematic review was performed using the protocol for systematic reviews that we published in 2015 (224). The protocol had been prospectively registered: International Prospective Register for Systematic Reviews (PROSPERO) number CRD42014013523. However, the publication period was extended to include 2016 (Jan 2000 to Dec 2016) (224).

Literature search

The Medline and Scopus databases were searched for relevant studies following the research strategy outlined in the protocol (224), using the key phrases ‘vitamin D’, ‘endothelial function’, ‘inflammation’ and ‘randomised (randomized) control trial’. Using keywords identified in this initial search, a broader secondary search of Medline, Science

Direct, Scopus, Web of Science, Cochrane Library of Systematic Reviews, ProQuest, Wiley and Highwire Press was conducted.

Data Extraction

This broader search yielded a total of 4,625 studies. Following the removal of duplicates, 3,479 studies were screened by title and abstract. All non-RCTs or RCTs that had durations of <12 weeks were excluded. Following screening and the application of inclusion and exclusion criteria, 3,459 studies were removed, leaving a total of 20 studies.

Quality Assessment

The full text of each of these studies was reviewed individually, including examination of the reference list of each paper and six studies were subsequently excluded (see Fig. 1 for reasons). Determination of study quality for inclusion was conducted by three independent investigators (AA, EKC, MJS) to minimise bias, and was contingent on achieving a Jadad score of ≥3 (225, 226) and in addition allocation concealment and appropriate data analysis. After applying these criteria, 14 RCTs remained in the systematic review. Study selection was conducted in accordance with PRISMA guidelines (227), and is illustrated in Figure 5.

Statistical Analysis

For studies in which the same methods for measuring endpoints were used, pre-intervention, post-intervention and mean change outcomes for endothelial dysfunction were reported. The mean change was calculated in studies with parallel design by subtracting the mean effect in the placebo (control) group from the mean in the treatment group. Raw data from both the placebo and treatment groups were used to calculate standard deviation (SD) using standard error (SE) and confidence interval (95% CI) values for each of the studies. Meta-analysis was unable to be conducted due to the variation in endothelial dysfunction measurements between studies.

Results

Study characteristics

Following our systemic review protocol, 14 RCTs were comprehensively reviewed and data extracted. Fourteen (n=14) RCTs were analysed: twelve parallel double-blinded and two single-blinded placebo controlled studies. The mean RCT duration was 29.14 weeks (max=52, min=16, range=36). Four studies each were conducted in the United States (US) and United Kingdom (UK), and one study was conducted each in Australia, Denmark, Hong Kong, Ireland, Israel and Korea. Across the review, a total of 1,650 participants (max=305, min=47, mean=118/study) were assessed comprising a mean of approximately 34% males and 58% females per study. The age of participants varied from 18 to 76 (range=58) with a mean age of 58 years, and the body mass index (BMI) across the cohort was 29.29 kg/m^2 (overweight bordering on obese) (see Table 5). Among those, six study arm populations started the trial with 25(OH)D less than 50 nmol/L and ended with levels greater than 75 nmol/L (see Table 6).

As indicated in Table 5, the effect of cholecalciferol supplementation on EF was evaluated in fourteen studies: four of these studies assessed healthy participants (134, 183, 228, 229); four studies assessed diabetic patients (177, 211, 230); three studies assessed pre-diabetic participants with confirmed hypovitaminosis D (231-233); one study assessed hypertensive individuals (174); and the last study assessed participants with a previous history of myocardial infarction (175). Vitamin D dose varied substantially across the studies. The lowest dose administered was 200 IU per day (228) and the highest was 7,000 IU/day (Wamberg et al., 2012). Forouhi et al (2016) administered one single 100,000 IU dose per month for four months, whereas Witham et al 2010 used only one initial single dose of either 100,000 IU vitamin D3 or 200,000 IU vitamin D3 (177). The commonest route of administration was per oral (PO) tablets (173, 174, 212, 230, 231, 234), followed by PO solution (175, 177, 233), PO capsules (211, 228, 229, 232), and PO fortified biscuits (183). All studies used cholecalciferol (D_3) supplements.

EF was assessed via various methods. Of those RCTs using physiologically induced outcomes, four used FMD (174, 177, 183, 212), five studies used PWV (173, 174, 183, 230, 233) and three used AIx (183, 211, 230). Other parameters measured were CRP (173-175, 183, 211, 228-234), IL-6 (229, 231, 232, 234), adiponectin (211, 232, 234), leptin (211, 234), TNF-α (175, 231, 232), plasminogen activator inhibitor-1 (PAI-1) (231, 234) sICAM (229), endothelial progenitor cells (173), reactive hyperaemia, E-selectin and Von Willebrand Factor (VWF) (174), monocyte chemoattractant protein-1 (MCP-1) (234) and matrix metallopeptidase 9 (MMP-9) (228).

Quality analysis

In response to vitamin D supplementation, the majority - 11 of the RCTs - demonstrated non-significant changes in EF, while only three RCTs demonstrated a statistically significant improvement in EF (Forouhi et al 2016 (233), Breslavsky et al 2013 (211) and Harris et al 2011 (212)). Only the study by Witham et al (2013a) (175) detected an effect of vitamin D supplementation on systemic inflammatory outcomes, namely a significant decline in systemic inflammation, as indicated by a reduction in CRP in the treatment group (174) (see Table 6). The remaining studies (11 RCTs) did not find any relationship between circulating 25(OH)D and endothelial cell activation or systemic inflammatory molecules.

Eleven studies concealed from their participants whether they were allocated to the testing or placebo groups and blinded data analysis, establishing Jadad scores of 5. Harris et al (2011) has a Jadad score of 4/5 with a sufficient allocation concealment and data analysis (212). Meanwhile, Breslevsky et al. (2014) lacked appropriate description of sequence randomisation and did not complete adequate allocation concealment leading to a Jadad score of 4/5 (211). Sinha-Hikim et al (2015) was allocated a Jadad score of 3/5 due to insufficient information of withdrawal rates and a lack of blinded description of randomisation (231). Collectively, 13 RCTs examined had minimal risk of bias with Jadad scores of 4/5 and above, while one RCT had a Jadad score of 3/5.

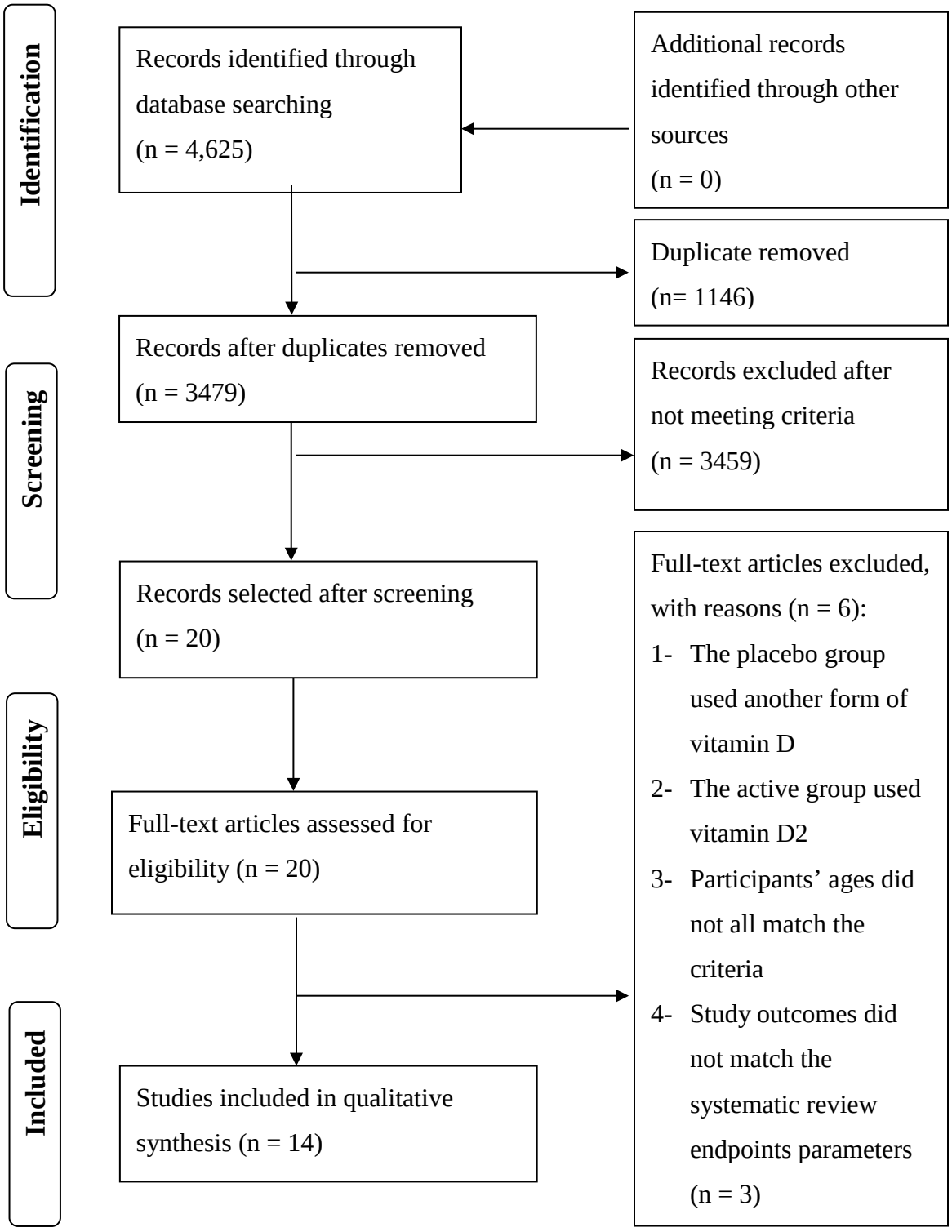

Figure 5: PRISMA flow diagram of study selection for the systematic review of vitamin D supplementation and endothelial function.

Table 5: Demographic data of RCTs in the systematic review.

Authors	Year	Vitamin D dose (IU/d)	Duration (wk)	Population	Location	n	Age (y)	% Male	% Female	BMI	Study Outcomes
Witham et al (177)	2010	100,000/200,000 IU in the start only	16	Diabetic	UK	61	57 - 76.4	67.2	32.8	32.3	FMD
Harris et al (212)	2011	2143	16	Pre-diabetic African-American	US	70	19 - 50	46.5	53.5	29.8	FMD, PTH
Gepner et al (183)	2012	2500	16	Post-menopause women	US	14	60 - 66	0	100	26.2	AIx, PWV, FMD & CRP
Wood et al (229)	2012	400 or 1000	52	Healthy postpartum women	US	305	61.6 - 66	0	100	26.7	Hs-CRP, sICAM, IL-6
Muldowney et al (228)	2012	200, 400 or 600	22	Healthy adult	Ireland	202	20 - 40	2.9/35.9	77.1/ 64.1	26.1	Hs-CRP and MMP-9
Yiu et al(173)	2013	5000	12	Diabetic	HK	100	57 - 73	50	50	25.5	PWV, hs-CRP and endothelial progenitor cells
Wamberg et al(234)	2013	7000	26	Healthy adult	Denmark	52	18 - 50	29	71	40.4	Hs-CRP, IL-6, MCP-1, Adiponectin, leptin, PAI-1
Witham et al(175)	2013a	1789	26	PMHx of myocardial infarction	UK	75	56 - 76	59.3	40.7	-	Reactive hyperemia, CRP, E-selectin, TNF-α, VWF
Witham et al(174)	2013b	3571	52	Hypertensive	UK	159	>70	51.5	48.5	28.2	PWV, FMD, CRP
Breslavsky et al (211)	2013	1000	52	Diabetic	Israel	47	56.1 - 76	46.8	53.2	29.3	AIx, leptin, adiponectin and hs-CRP
Ryu et al (230)	2014	2000	24	Diabetic	Korea	62	30 - 69	-	-	24.8	Aix, PWV, hs-CRP
Gagnon et al (232)	2014	4000	26	Pre-diabetes or at-risk of diabetes	Australia	95	41.9 - 66.4	31	69	31.5	Adiponectin, CRP, TNF-α and IL-6
Sinha-Hikim et al (231)	2014	Varied	52	Pre-diabetes & hypovitaminosis D	US	80	43.9 - 59.1	30	70	32.7	IL-6, TNF-α, hs-CRP and PAI-1
Forouhi et al (233)	2016	100,000/ Month	16	Pre-diabetes or at-risk of diabetes	UK	228	43.9 - 61	30	70	_	PWV, CRP

FMD: follow mediated dilatation, PWV: pulse wave velocity, AIs: augmentation index, RHIx: reactive hyperaemia index, hs-CRP: high sensitive C-reactive protein, TNF-α: tumour necrosis factor alpha, IL-6: interleukin-6, MMP-9: matrix metallopeptidase 9 and PAI-1: plasminogen activator inhibitor-1.

Table 6: Outcomes of RCTs evaluating the effect of cholecalciferol supplementation upon EF and systemic inflammatory biomarkers.

Author & year	Vitamin D_3 dose (IU/d)	Administration	Jadad score (allocation concealment/data analysis)	25(OH)D conc. (nmol/L)		Significant treatment effects				Endothelial cell activation molecule / systemic inflammation molecule	Conclusions
				Initial	Final	FMD	PWV	AI_x	RHIx		
Witham et al 2010 (177)	993	PO solution	5 (Yes/Yes)	41.00	59.00	No	-	-	-	-	No effect on EF
Witham et al 2010 (177)	1786	PO solution	5 (Yes/Yes)	48.00	76.00	No	-	-	-	-	No effect on EF
Harris et al 2011(212)	2143	PO tablet	4 (Yes/Yes)	34.30	100.90	Yes	-	-	-	-	Beneficial effect on FMD
Gepner et al 2012 (183)	2500 IU/day	PO food	5 (Yes/Yes)	75.63	114.83	No	No	No	-	No effect on CRP	No effect on EF or inflammation
Wood et al 2012 (229)	1000 IU/day	PO capsule	5 (Yes/Yes)	32.41	75.66	-	-	-	-	No effect on hs-CRP, IL-6 nor sICAM	No effect on EF or inflammation
Wood et al 2012 (229)	400 IU/day	PO capsule	5 (Yes/Yes)	32.74	64.86	-	-	-	-	No effect on hs-CRP, IL-6 nor sICAM	No effect on EF or inflammation
Muldowney et al 2012(228)	200, 400, 600 IU/day	PO capsule	5 (Yes/Yes)	70.00	79.00	-	-	-	-	No effect on hs-CRP, MMP-9	No effect on biomarker of CVS health
Yiu et al 2013(173)	5000 IU/day	PO tablet	5 (Yes/Yes)	52.75	146.50	-	No	-	-	No effect on hs-CRP	No effect on EF or hs-CRP
Wamberg et al 2013 (234)	7000 IU/day	PO tablet	5 (Yes/Yes)	34.50	110.20	-	-	-	-	No effect on any inflammatory markers (hs-CRP, MCP-1, IL-6, adiponectin, leptin, MMP-9 and PAI-1)	No effect on inflammation
Witham et al 2013a (175)	1789 IU/day	PO solution	5 (Yes/Yes)	49.00	62.00	-	-	-	No	There is a decline in CRP only	Anti-inflammatory but not EF effect
Witham et al 2013b (174)	3571 IU/day	PO tablet	5 (Yes/Yes)	45.00	67.50	No	No	-	-	No effect on any of endothelial function measurement	No effect on EF
Breslavsky et al 2013(211)	1000 IU/day	PO capsule	4 (No/No)	29.50	44.00	-	-	Yes	-	No effect on leptin, adiponectin or hs-CRP	Beneficial effect on EF

* Vitamin D3 dose calculated as follows: (100 - baseline vitamin D3) x weight (kg) x 15.7 IU/week. PO: per oral, FMD: follow mediated dilatation, PWV: pulse wave velocity, AIs: augmentation index, RHIx: reactive hyperaemia index, hs-CRP: high sensitive C-reactive protein, TNF-α: tumour necrosis factor alpha, IL-6: interleukin-6, MMP-9: matrix metallopeptidase 9 and PAI-1: plasminogen activator inhibitor-1.

Table 6: Outcomes of RCTs evaluating the effect of cholecalciferol supplementation upon EF and systemic inflammatory biomarkers (cont.).

Author & year	Vitamin D_3 dose (IU/d)	Administration	Jadad score (allocation concealment/data analysis)	25(OH)D (nmol/L)		Significant treatment effects				Endothelial cell activation molecule / systemic inflammation molecule	Conclusions
				Initial	Final	FMD	PWV	AI_x	RHIx		
Ryu et al 2014(230)	2000 IU/day	PO tablets	5 (Yes/Yes)	30.75	86.50	-	No	No	-	No effect of hs-CRP	No effect on hs-CRP nor EF
Gangnon et al 2014(232)	4000 IU/day	PO capsule	5 (Yes/Yes)	47.00	95.00	-	-	-	-	No effect on Adiponectin, CRP, TNF-alpha or IL-6	No effect on inflammatory biomarkers
Sinha-Hikim et al 2014(231)	*	PO tablet	3 (Yes/No)	54.75	175.00	-	-	-	-	No effect on IL-6, TNF-alpha, hs-CRP nor PAI-1	No effect on inflammatory biomarkers
Forouhi et al 2016 (233)	100,000 IU/Month	PO solution	5 (Yes/Yes)	45.80	51.20	-	Yes	-	-	No effect on CRP	Beneficial effect on PWV and no effect on CRP

* Vitamin D3 dose calculated as follows: (100 - baseline vitamin D3) x weight (kg) x 15.7 IU/week. PO: per oral, FMD: follow mediated dilatation, PWV: pulse wave velocity, AIs: augmentation index, RHIx: reactive hyperaemia index, hs-CRP: high sensitive C-reactive protein, TNF-α: tumour necrosis factor alpha, IL-6: interleukin-6, MMP-9: matrix metallopeptidase 9 and PAI-1: plasminogen activator inhibitor-1.

Discussion

Vitamin D and endothelial function (EF)

This systematic review found a positive causal association between cholecalciferol supplementation and EF in three of the RCTs that met our criteria (211, 212, 233). However, in 11 of the RCTs no significant relationship was found between 25(OH)D and EF.

No significant effect on EF was observed in seven trials wherein the participants had an overall average starting mean level of 25(OH)D ≥ 50 nmol/L (173, 175, 177, 183, 228, 231, 232). For the three studies in which a positive effect on EF was found, 25(OH)D levels were initially below 50 nmol/L at 29.5, 34.3 and 45.8 nmol/L in the studies by Breslavsky et al (2013) (211), Harris et al (2011) (212) and Forouhi et al (2016) (233) , respectively. For the other five trials where initial 25(OH)D levels were < 47 nmol/L, supplementation had no effect on EF (174, 177, 229, 230, 234). The Witham et al (2010) study was comprised of two trials with slightly different initial 25(OH)D levels at 48 nmol/L and 41 nmol/L but used different doses of vitamin D. Again, there was no observable relationship between the study durations and the endothelial function outcomes.

Regarding the three studies which found 25(OH)D to have an effect on ED, Harris et al used per oral (PO) tablets (2,143 IU/day), Breslavsky et al (2013) used PO capsules (1,000 IU/day) and Forouhi et al (2016) used PO solution (100,000 IU/month). All three of these studies were conducted on populations comprised of diabetic (211) and pre-diabetic (212, 233) subjects. One of the studies on pre-diabetic subjects had a mean baseline level of 25(OH)D consistent with hypovitaminosis D (212). The three studies used different methods of EF analysis, namely FMD, PWV and AIx. Two had a study period of 16 weeks (212, 233) and one had a study period of 52 weeks (211). It is worth mentioning here that inclusion of participants with initial 25(OH)D < 50 nmol/L is important as this allows a substantial change in 25(OH)D level which in turn, makes it more likely that a relationship between 25(OH)D level and EF is found. Also, different form of vitamin D supplementation might affect the end point level of 25(OH)D and participants' medical history (for example diabetes or pre-diabetes) also plays a vital role.

Harris et al (2011) utilised 2,143 IU/day of vitamin D delivered orally in tablet form. This study was of high quality as determined by the Jadad score (4/5). Vitamin D3 supplementation above 2000 IU a day for 16 weeks increased the level of 25(OH)D from 34.2 nmol/L to the very high level of 100.9 nmol/L. In contrast to this, over the same period of time the placebo group increased from a 25(OH)D level of 38.2 nmol/L to the moderate, but still insufficient, level of 48.7 nmol/L. As measured by FMD, a significant improvement in EF was recorded in the treatment group compared

to the placebo group. The authors concluded that 25(OH)D supplementation had a beneficial effect on EF because a significant vasodilatory response was achieved. Indeed, the African American population examined are at high risk of hypertension, shown to relate to a multiplicity of intimately related minor genes (235). Moreover, this population is at increased risk of hypovitaminosis D (235). Baseline data showed that BMI was high (30.4 m^2/kg) in alignment with the remaining RCTs examined. In contrast to most of the other studies, the baseline data indicated one of the lowest initial values for mean 25(OH)D. This may be explained by the Gc phenotype of the vitamin D binding protein (Vitamin DBP), prevalent in people with darker skin, which has a higher affinity for 25(OH)D and thus leaves lower amounts of unbound 25(OH)D in the blood (1). This indicates that circulating 25(OH)D values, may not accurately represent the vitamin D status of people with darker skin, which may explain the more significant effect of vitamin D3 supplementation on endothelial function (1).

Breslavsky et al (2013) examined EF using AIx in diabetic patients and reported that 1,000 IU/day of cholecalciferol supplementation was correlated with improved AIx scores as a measure of enhanced EF. The study design implemented was of lower quality compared to the majority of RCTs we assessed. This was due to inadequate allocation concealment and unclear reporting of the randomisation sequencing. In alignment with the outcomes of Harris et al (2011), the study demonstrated an insufficient final 25(OH)D value (44 nmol/L), suggesting that this vitamin D dose may not have achieved a pharmacologically significant effect.

Three of the RCTs evaluated the effect of cholecalciferol supplementation on EF in pre-diabetic participants. The study by Forouhi et al (2016) had a large sample size of 228 participants compared to the sample sizes of Gagnon et al (2014) and Sinha-Hikim et al (2014), which assessed 95 and 80 participants, respectively (231, 232). The studies by Forouhi et al (2016) and Gagnon et al (2014) study had perfect Jadad scores of 5/5 while the study by Sinha-Hikim et al (2014) had a lower Jadad score of 3/5. The RCT by Forouhi et al (2016) directly assessed endothelial function through PWV and found that cholecalciferol supplementation improved EF. The RCTs by Gagnon et al (2014) and Sinha-Hikim et al (2014) did not directly assess endothelial function; rather, they only assessed the inflammatory biomarkers and did not find any effect of cholecalciferol supplementation. Forouhi et al (2016) also examined the inflammatory biomarker C-reactive protein (CRP), where there was no effect after cholecalciferol supplementation. The Forouhi et al (2016) study was the only RCT design incorporating a single large dose (100,000 IU) per month.

Effect of obesity and T2DM

In this systematic review the average BMI across the studies was mostly over 25kg/m^2, which parallels a previous meta-analysis of vitamin D and EF that demonstrated a proportional trend of vitamin D effects in higher BMI subjects (222). As adipocytes express the VDR, it is possible that increased adiposity might cause increasing sequestration of 25(OH)D (236). Other studies have demonstrated that an increase in circulating 25(OH)D is associated with reduced adiposity [weight loss] (236). A previous meta-analysis found that BMI weakly modifies the effect that vitamin D supplementation has on EF (236). However, the present systematic review could not find any relationship between low BMI and an improved effect of cholecalciferol on EF.

The previously mentioned meta-analysis also found vitamin D supplementation to have the greatest effect on EF in T2DM patients where the initial 25(OH)D levels were the lowest (236). The studies included in this review which had T2DM and pre-diabetic patients with very low initial mean 25(OH)D levels yielded mixed results, with some of these studies revealing an improvement in EF (211, 212, 233) and others reporting no such improvement (173, 177, 230-232).

Taking into consideration the relationship between EF and insulin resistance (IR) it is likely that the effect of cholecalciferol supplementation in diabetics compared to non-diabetics may differ. Indeed, this relationship may also interact with adiposity. Previous studies show that decreased endothelium-derived nitric oxide (NO) is the central link between endothelium and IR (29, 55). Vascular endothelial destruction pathognomonic of diabetes mellitus and subsequent endothelial dysfunction is linked to a reduction in NO-dependent vascular activity (29). Furthermore, comorbidities linked to both obesity and diabetes, such as atherosclerosis, may reduce endothelial-derived NO (11, 45, 49). These factors may explain less reactivity in both pre-diabetic and diabetic cohorts. However, the distinction in outcomes among studies examining the cholecalciferol impacts on EF in cohorts with the same disease status (i.e. diabetics vs. diabetics; pre-diabetics vs. pre-diabetics) is unexpected. However, it is noted that these studies were conducted in different populations and used different methods of EF analysis.

The three RCTs which found a positive correlation between cholecalciferol supplementation and EF outcomes had an initial 25(OH)D mean indicating mild vitamin D deficiency (25-50 nmol/L). Two of those studies achieved the lowest final mean 25(OH)D values of the RCTs reviewed. In fact, these results suggest a borderline sufficiency of 25(OH)D is enough to improve EF, according to the current recommended cut-off level of 50 nmol/L (237). However, the third study achieved 25(OH)D >75 nmol/L, which could be the future cut-off for the extra-skeletal benefits of 25(OH)D.

Vitamin D and biomarkers of systemic inflammation

Of the 11 studies that examined biomarkers of systemic inflammation, only one study demonstrated a positive association between reduced systematic inflammation measured via CRP and 25(OH)D (175). Notwithstanding this, other studies using CRP as an inflammatory marker showed no correlation (170, 183, 229, 234). Interestingly, the study cohort which elicited the significant reduction in CRP was hypertensive prior to the administration of cholecalciferol. While CRP is a nonspecific acute phase reactant, it has been linked to hypertension (238). No other studies using CRP as an inflammatory biomarker were conducted on hypertensive patients, so unfortunately this effect cannot be compared in similar cohorts.

One would suspect that 25(OH)D sufficiency, which is linked to lower inflammatory states (33), might demonstrate a less marked reduction in inflammatory biomarkers such as CRP, as these are constantly being released at higher rates in hypertensive patients. This systematic review supports this concept. The studies which assessed those populations that are also assumed to have increased inflammatory stress, such as diabetic and pre-diabetic patients, did not show any reductions in inflammatory biomarkers.

Two strong RCTs examining endothelial cell activation molecules E-selectin (175) or ICAM (229) did not show a significant reduction. Cholecalciferol supplementation was shown to have little impact on the markers of endothelial function.

Limitations of this systematic review

The studies were conducted on populations of varying demographics, including the absence or presence of comorbidities (which included pre-diabetes, T2DM and CVD), the types of comorbidities, ethnicity, gender, BMI and age, and thus it is difficult to directly compare the results between the groups. Furthermore, the studies measured different outcomes, namely different types and combinations of inflammatory biomarkers, and used different techniques to evaluate EF. Also, the studies varied in duration from 12-52 weeks and in the cholecalciferol dosage amounts, frequencies and administration methods. In addition, some studies did not specifically investigate EF or endothelial cell activation molecules but rather the biomarkers of systemic inflammation as a group.

Unfortunately, meta-analysis could not be performed on this dataset for several reasons. The primary issue was missing data despite numerous unsuccessful attempts to contact the authors. Studies that made it through the selection process utilised different methodological approaches, rendering it

impossible to appropriately apply a meta-analytic approach. Furthermore, missing information in some studies (173, 212) led to insufficient FMD data on which to conduct a meta-analysis. Despite having five PWV studies included in the systematic review (173, 174, 183, 230, 233), each study implemented a different PWV protocol. Only one study examined EF using several PWV approaches, which were heart-carotid PWV, heart-ankle PWV and brachial ankle PWV (173). Meanwhile, other RCTs conducting PWV analysis used singular approaches that could not be evaluated by meta-analysis (174, 183, 230, 233). Only two RCTs assessed EF using the endothelium-specific biomarkers sICAM (229) and E-selectin (175).

In this systematic review five studies (228, 229, 231, 232, 234) assessed endothelial function using endothelial cell biomarkers without physical assessment of endothelial function. These biomarkers include CRP, IL-6, ICAM-1, MMP-9, PAI-1,, VCAM-1 MCP-1, E-selectin, p-selectin and TNF-α. The aetiology of endothelial dysfunction generally involves endothelial cell activation (239). Therefore, identifying when the endothelial cells have been activated can be important in the early detection of disease. The activated endothelial cells are permeable, pro-inflammatory and pro-thrombotic (239). Inflammatory activation of endothelial cells leads to increased levels/expression of E-selectin, P-selectin, sVCAM-1, sICAM-1, TNF-a, CRP, IL-6 and MCP-1 (239). One study found levels of IL-6 and E-selectin to correlate with FMD results, while another study found CRP and PAI-1 to have an inverse relationship with FMD (240, 241). Endothelial cells activation molecules and systemic inflammatory molecules indicate endothelial dysfunction through its relationship to endothelial cells. This relationship will be only a marker of this dysfunction and not a definitive interpretation of endothelial function status. For more discussion of the link between these molecules and endothelial function see vitamin D and endothelial function paper (26).

Conclusion

This systematic review provides insufficient evidence to support the causal role of cholecalciferol supplementation in improving EF and suppression of systemic inflammation and endothelial cell activation molecules. Nonetheless, the results of this systematic analysis suggest that the use of cholecalciferol supplementation to overcome vitamin D deficiency may serve to improve EF in individuals with pre-diabetes or T2DM, and to lower the systemic inflammatory response in individuals with hypertension. Considering the complex interrelationships between IR, obesity and endothelial cells, the potential for cholecalciferol supplementation to augment EF in the diabetic population and to relieve systemic inflammation in the CVD population requires further investigation with high quality studies, and to ensure adequate dosage to improve circulating 25(OH)D in the blood.

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Chapter 3:

General Materials & Methods

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1 Materials

1.1 Blood centrifuge:

Blood was centrifuged at 3000 revolutions per minute (rpm) for 10 minutes (242) using MPW-53 ™ (MPW MED. INSTRUMENTS, Poland).

1.2 Blood Collection Set:

BD Vacutainer® Safety-Lock™ Blood Collection Set with tube holder (Becton, Dickinson and Company. ©2006 Franklin Lakes, NJ 07417).

1.3 Blood collection tubes:

BD Vacutainer® Heparin Tubes are spray-coated with either lithium heparin or sodium heparin and are used for plasma determinations in chemistry (Becton, Dickinson and Company. ©2006 Franklin Lakes, NJ 07417).

1.4 Blood pressure:

Blood pressure was measured using semi-automated blood pressure monitor (IA2 model, OMRON Healthcare Com. Ltd. Japan ™, Head office in The Netherlands).

1.5 Dual energy x-ray absorptiometry (DEXA):

Lunar Prodigy (GE Medical Systems Lunar Madison, Wisconsin USA).

3.1.6 Hs-CRP measurement device:

Hs-CRP has been measured in the lab using the QuikRead go ™ (Orion Diagnostica Oy, Finland). It is a photometer which we used to measure the absorbance of the cuvette and convert this into a concentration value.

1.7 Medi-swab alcohol swabs:

Smith and Nephew, Mount Waverley, VIC, Australia

1.8 Multianalyte of biomarkers using Luminex xMAP® technology (MAGPIX®):

This technique involves the use of fluorescent-coated magnetic beads (MagPlex®-C microspheres) which each have a specific magnetic microsphere and capture antibody coating, indicated by the fluorescent code assigned to each one. Plasma is then added to the beads, and this mixture is then incubated with Streptavin-PE conjugate to complete the reaction. The beads then emit a fluorescent signal to report the plasma levels of these biomarkers: necrosis factor-alpha (TNF-α), soluble vascular adhesion molecules (sVCAM), soluble intracellular adhesion molecules (sICAM), leptin, hepatocyte growth factor (HGF), C-reactive protein (CRP), D-dimer, P-selectin, and myeloperoxidase (MPO).

1.9 Pulse Contour Analysis (PCA) (Pulse Trace-1000, MicroMedicals, UK):

The photoelectric plethysmograph is an infra-red transmitting probe which is clipped to the index finger of the right (55). Here it measures the amount of light absorbed by the haemoglobin, which is proportional to the volume of blood present in the finger and thus indicates the pulse. It is also connected to an analogue to digital convertor which records the pulse waveforms by measuring the transmission of infra-red light through the finger for 15 - 30 seconds to produce a typical digital volume pulse (DVP), from which stiffness index (SI) and reflective index (RI) are derived.

SI is a measure of large artery stiffness, where SI = PPT (sec)/ height (m). The normal SI for adults varies between 5 and 10 m/s. RI measures small artery vascular tone and thus indicates peripheral vasodilation; it is determined by the ratio of the amplitude (heights) of wave B to wave A. Vasodilation leads to a smaller RI while vasoconstriction results in a rise in its value. Factors such as exercise or caffeine intake which influence peripheral vascular tone may influence RI. RI varies between 60-90% in normal subjects. Hence the Pulse Trace System analysis of DVP is simple and its results are strongly correlated with the augmentation index and central pulse wave velocity (55).

2 Methods:

2.1 Participants and experimental Protocol:

Participants were recruited by various methods, including the distribution of flyers, local community newspapers and a radio advertisement (Curtin University Radio). Prospective participants completed a screening form (Appendix 2). Then each applicant was assessed to ensure that that the participants would meet the specific selection criteria. These criteria included the following: Australian of European descent or Middle Eastern; adults of at least 18 years of age; a constant weight (± 2 kg) over the last six months; not suffering from any medical conditions involving the thyroid, liver, kidney or heart; not diagnosed with Type 1 diabetes; not suffering from any current illness or infection requiring antibiotics; not experiencing gastrointestinal problems and did not have histories of gastrointestinal surgeries; not on any medication or supplements (Vitamin D, fish oil or thiamine supplements), nor on any special or commercial dieting programs that may affect the body's metabolism; and not taking part in strenuous physical activities (subjects engaged in competitive sports, team games, running (4.3 min/km) or jogging (5.6 min/km) were excluded) (243). All participants were informed by phone that they have been chosen for the study. They were then asked to come for an introductory visit to learn more about the place they would attend on the experiment day, and to be given the food they were to eat for dinner the night before the experiment. Also, participants were given parking vouchers and signed the consent forms after reading the study protocol.

Thereafter, participants underwent a full-day experiment which began by resting in the lab for half an hour, then taking basal measurement and blood, after which glucose tolerance test were performed for two hours and endothelial measurements were taken throughout. Finally, another blood sample was collected at 2 h and then participants' body compositions were assessed with the DEXA and BIA machines.

2.2 Measurement of Participant Characteristics:

2.2.1 Blood collection:

Venous blood samples were taken from participants after half an hour of lying down in the supine positon. Blood was drawn into specimen tubes (vacutainers) containing an EDTA (plasma) green top with a gel separator. After blood collection, tubes were placed in the centrifuge for 10 minutes. Blood plasma was collected by disposable transfer pipettes in sealed tubes, after which each participant's plasma was stored at -80˚C for later analysis.

2.2.2 Blood analysis:

Blood analysis was conducted in two locations within Perth, Western Australia: at School of Public Health laboratories, Curtin University or the pathology departments of Fiona Stanley Hospital. At the School of Public Health in Curtin University, we measured the inflammatory markers hsCRP, TNF-α, HGF, P-Selectin, sVCAM, sICAM, and leptin using A MAGPIX system (Merck Millipore, Luminex, Austin, TX, USA). The QuikRead go ™ device (Orion Diagnostica Oy, Finland) was used to measure hsCRP. In the pathology departments at Royal Perth Hospital and Fiona Stanley Hospital, 25(OH)D, PTH, triacylglycerol (TGA), total cholesterol, and lipid fractions were measured. More details of each blood analysis will be given in the following chapters.

2.2.3 Blood pressure:

Participants were measured under standardized conditions, namely an overnight fast of at least 12 hours, and having rested in a supine position for at least half an hour. Participants were measured at baseline (after the half hour rest) before the experiment and then every fifteen minutes for two hours thereafter. Blood pressure was taken twice by the Omron blood pressure machine (Model T8, Omron, Japan) and the mean blood pressure was calculated.

2.2.4 Body composition:

Body composition (fat mass, fat-free mass, body water and skeletal mass) was assessed by:
dual energy x-ray Absorptiometry (DEXA), Prodigy Model (Lunar Corporation, USA).

Bioelectrical Impedance Analysis (BIA) using both the Direct Segmental Multi-frequency Bioelectrical Impedance Analysis Method (DSM-BIA) (General Electric Company, Asia) and the medical Body Composition Analyzer (mBCA) (Seca, Germany).

These were performed by a licensed investigator (candidate) at the Curtin University outpatient physiotherapy clinic. Each participant was asked to only wear a surgical gown and their underwear, and to remove any metal they may have been wearing, where it was possible to do so.

2.2.5 Body height:
Height was measured with the participant standing, using a stadiometer fixed to the wall, and this was recorded to the nearest 0.1 cm.

2.2.5 Body weight:
Body weight of each participant was measured after an overnight fast, while wearing a light surgical gown, on a digital scale (Tanita System 502, Tokyo, Japan), and this was recorded to the nearest 100g.

2.2.6 Waist circumference:
Waist circumference was measured as described by Norton and Olds (1996) (244). The waist measurement was taken at the level of the narrowest point between the lower costal (rib) border and the iliac crest. If there was no obvious narrow point, then the measurement was taken at the mid-point between these two landmarks. Each measurement was taken with the arms relaxed by the sides. The waist measured twice and a mean value recorded for each participant.

2.2.7 Metabolic Syndrome (Mets) Assessment:
MetS status was determined using the recent criteria published by Alberti et al (2009) (245). These criteria include having three of the following elements: elevated waist circumference, elevated triglyceride, reduced high density lipoprotein (HDL), increased blood pressure and elevated glucose in the blood (245).

2.2.8 Measurement of insulin resistance:
The gold standard method for the measurement of insulin resistance is the “hyperinsulaemic-euglycaemic clamp technique” in which simultaneous infusion of fixed doses of insulin and variable doses of glucose infusion are carried out in order to assess the individual response to insulin (246). However, at the clinical level an oral glucose tolerance test (OGTT) is a well-established alternative and is the method preferred by World Health Organization (WHO) and Australian expert committees (247). In this thesis, we evaluated participants’ insulin resistance using the OGTT. This involved

measuring the participants' glucose and insulin levels were in fasting status before oral administration of 75g of glucose, and then again two hours after ingestion.

Finally, we evaluated the homeostasis model assessment of insulin resistance (HOMA-IR), quantitative insulin-sensitivity check index (QUICKI), McAuley's index (248) and Sluiter et al equation (249) to detect insulin resistance. Although there are many useful indices of insulin resistance, these three are in common use. QUICKI and HOMA-IR depend on the fasting measurement of insulin and glucose. McAuley's index measures fasting insulin and triglyceride levels and as such, the specificity of this index is well accepted. McAuley's index considers a fasting insulin level greater than 12.2 mU/l in normoglycaemic individuals to be a highly specific test for insulin resistance.

HOMA-IR = Fasting insulin (U/L) x fasting glucose (mmol/L) / 22.5 (250).
QUICKI = 1/ [log fasting insulin (mU/L) + log fasting glucose (mg/dl)] (251).
McAuley's index = Mass fat free (Mff)/I= exp [2.63 – 0.28 ln (insulin) – 0.31 ln (triglycerides)] (252).
Sluiter et al equation for ISI= 10000(Insulin *glucose) (249)

2.2.9 Physical activity checklist – International Physical Activity Questionnaire (IPAQ):

We estimated the normal physical activity levels of the participants using the IPAQ, which measures the activities participated in over the last seven days of their lives (Appendix 1). The participants indicated the times spent on each activity within the following categories: strenuous, moderate, walking and sitting. As a result, we calculated the overall physical activity levels of the participants and categorised them into three groups according to their levels of physical activity: low, moderate and high.

2.2.10 Measurement of endothelial function using Pulse Contour Analysis (Micro Medicals):

Endothelial function was assessed by photoplethysmography through the Pulse Trace-2000 (MicroMedicals, UK) (Figure 6). A small photoplethysmograph, which is a unit that transmits infra-red light, was attached to each participant's finger and from there it recorded the blood pressure waveform (55). The analogue-to-digital convertor then used this data to generate a digital volume pulse (DVP) which duplicated the behavior and characteristics of the waveform (55). Each measurement cycle lasted for 20 seconds and was comprised of at least 10 pulse waves. Every 15 minutes, we measured pulse wave velocity two times (>20 waves in total) within two minutes to represent that period of time (253-255). This technique produced reliable results with an intra-

individual variability of SI (CV = 9.9%) and RI (CV = 3.8%) (256). Its external validity has been described as good (253-255).

This method was used to measure the heights of and times between the systolic and diastolic inflections, which were then used in conjunction with the height of the participant to calculate the stiffness index (SI = artery stiffness), reflective index (RI = systemic vascular tone) and pulse rate (PR). SI is calculated by dividing the height of the subject by the time between systolic and diastolic inflection peaks. RI is the percentage ratio of the height of the diastolic inflection to that of the systolic inflection. The DVP consists of a forward travelling wave and a backward travelling wave, with the latter arising from wave reflection. The first peak is related to the forward travelling wave (wave A) while the second one named “dichrotic notch” is linked to the backward travelling wave (wave B). In hypertensive diabetics, the second wave (reflected one) occurs sooner and overlaps with the first forwarding wave. Therefore, the second reflected wave is lost and peak to peak time (PPT) decreases. The consequence is a stiffer artery in hypertensive diabetic participants in comparison to the healthy individuals (55).

Figure 6: Overview of participant’s connection to PCA (PT-2000).

Chapter 4:

Paper:

The Association of Vitamin D Status with Dyslipidaemia and Biomarkers of Endothelial Cell Activation in Older Australians

Alyami, A. M., Lam, V., Soares, M. J., Zhao, Y., Sherriff, J. L., Mamo, J. C., ... & Coombes, F. (2016). The association of vitamin D status with dyslipidaemia and biomarkers of endothelial cell activation in older Australians. Nutrients, 8(8), 457. [Impact factor =3.759; cited by 1].

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nutrients

MDPI

Article

The Association of Vitamin D Status with Dyslipidaemia and Biomarkers of Endothelial Cell Activation in Older Australians

Ali M. Alyami [1], Virginie Lam [1], Mario J. Soares [1,*], Yun Zhao [2], Jillian L. Sherriff [1], John C. Mamo [1], Anthony P. James [1] and Fiona Coombes [3]

1 Health Promotion & Disease Prevention, School of Public Health, Curtin Health Innovation Research Institute, Faculty of Health Sciences, Curtin University, Perth 6102, Australia; alimahdim.alyami@postgrad.curtin.edu.au (A.M.A.); virginie.lam@postgrad.curtin.edu.au (V.L.); J.Sherriff@curtin.edu.au (J.L.S.); J.Mamo@curtin.edu.au (J.C.M.); T.P.James@curtin.edu.au (A.P.J.)

2 Occupation and the Environnent, School of Public Health, Faculty of Health Sciences, Curtin University, Perth 6102, Australia; Y.Zhao@exchange.curtin.edu.au

3 University Health Service, Support Services, Curtin University, Perth 6102, Australia; F.Coombes@exchange.curtin.edu.au

* Correspondance: m.soares@curtin.edu.au; Tel.: +61-8-9266-3220; Fax: +61-8-9266-2958

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Abstract: Background/Aims: Vitamin D has been investigated for many non-skeletal effects. The objective of this study was to determine whether circulating lipids, systemic inflammation, and biomarkers of endothelial cell activation varied with the vitamin D status of older Australians. Methods: One hundred and one participants were proportionately and randomly sampled across tertiles of 25 hydroxy vitamin D (25(OH)D) from a larger cohort of free living older adults (T1 median = 97; T2 median = 74.5; T3 median = 56.8 nmol/L). Overnight fasting blood samples were assayed for 25(OH)D, parathyroid hormone (PTH), insulin, triacylglycerol (TAG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C). Markers of systemic inflammation (high sensitivity C-reactive protein (hsCRP), tumour necrosis factor-α (TNF-α)) and endothelial activation (hepatocyte growth factor (HGF), P-selectin and soluble vascular cell adhesion molecule (sVCAM), soluble intracellular adhesion molecule (sICAM)) were determined. A general linear model multivariate analysis with a backward elimination procedure was performed. Results: Eighty-three participants (48 women, 35 men), aged 65 ± 7.7 years, BMI 28 ± 4.5 kg/m^2, with complete data were analyzed. The final parsimonious model controlled for age, gender, BMI, and McAuley's index, but excluded season, medications, and PTH. There were significant differences across 25(OH)D tertiles in TC (T1 < T3, p = 0.003; T2 < T3, p = 0.001), LDL-C (T1 < T3, p = 0.005; T2 < T3, p = 0.001), TAG (T2 < T3, p = 0.026), HGF (T1 > T3, p = 0.009) and sVCAM (T1 > T3, P = 0.04). Conclusions: Higher vitamin D status may protect the endothelium through reduced dyslipidaemia and increased HGF.

Keywords: vitamin D; endothelial function; inflammation; cardiovascular disease; lipids

1. Introduction

The 2011–2012 National Health Survey [1] revealed that most Australian adults were vitamin D sufficient, as judged by levels of 25 hydroxy vitamin D (25(OH)D) > 50 nmol/L, with only 23% having inadequate status. However, older Australians had a higher prevalence of inadequacy [1,2]. Contributing factors include limited sun exposure due to an increased awareness of skin cancer; skin pigmentation; genetic determinants; adiposity, illness, or immobility resulting in indoor living; age-related decline in the skin's ability to synthesize vitamin D; and dress habits [3,4].

The recommended level of 25(OH)D that signals vitamin D sufficiency is based on a value required for bone health [5], however there is growing interest in recommendations that optimize extra-skeletal outcomes, including cardiovascular health. The precise mechanism(s) whereby vitamin D status may modify the development of atherosclerosis are unknown [6], however there is evidence for roles in both endothelial function and plasma cholesterol levels [6]. The impact of vitamin D status on LDL-cholesterol (LDL-C) and total cholesterol (TC) remains equivocal due to the randomized controlled trials (RCT) published to date having small numbers of subjects, variable doses of vitamin D, a wide-range of intervention times, and a relative lack of participants with low vitamin D status at baseline [7].

The impact of inflammation on endothelial function is well recognized [8] and the understanding of the immune system's metabolism of vitamin D has exploded over the last five years [9]. Unlike many other cell types in the body, various immune cells can hydroxylate 25(OH)D, with the availability of 25(OH)D regulating the synthesis of the active form, 1,25 di-hydroxy vitamin D ($1,25(OH)_2D$ or calcitriol) rather than the hormonal regulation that characterizes renal synthesis [9]. Furthermore, unlike renal synthesis, there is a lack of negative feedback resulting in locally elevated levels of $1,25(OH)_2D$ [9]. Immune cells express vitamin D receptors (VDR) and can therefore respond to $1,25(OH)_2D$, although T cells need to be activated before the VDR gene is expressed [10]. In a similar manner, the endothelium expresses VDR and has the enzymatic machinery to locally produce the active hormone $1,25(OH)_2D$ from circulating 25(OH)D. Hence the endothelium also does not have a feedback control system, and synthesis of the hormone depends on the level of circulating 25(OH)D [6].

There are many systemic inflammation biomarkers and endothelial cell activation molecules that have been studied as surrogates for cardiovascular disease risk [11]. Commonly measured markers include tumour necrosis factor-alpha (TNF-α), high sensitivity C-reactive protein (hsCRP), leptin, soluble intracellular adhesion molecule (sICAM-1) and soluble vascular cell adhesion molecule (sVCAM-1), hepatocyte growth factor (HGF), and selectins (P and E-selectin), all of which are increased with the inflammatory state [12,13]. In general, higher levels of these biomarkers are considered to be independent risk factors for cardiovascular disease (CVD) [14], and vitamin D deficiency may lead to elevated levels of these biomarkers [13,15]. We and others have recently reviewed the data in the area and concluded that there is limited causative evidence to link 25(OH)D levels to systematic inflammation and endothelial dysfunction [6,13]. The purpose of this study is to investigate the potential links between dyslipidaemia, endothelial cell activation, and vitamin D status in older Australians.

2. Experimental Section

2.1. Subject Selection

One hundred and one participants were proportionately and randomly sampled across tertiles (T1 = highest, T2 = middle, T3 = lowest) of 25(OH)D from a larger cohort of free living older adults [16]. These participants were of European origin and residents of Perth, Western Australia. The primary study had ethical approval from the Curtin University Human Ethics Committee (approval number HR97/2011), and all participants provided informed written consent. The study was conducted between December 2011 and August 2012, a period that coincided with one of the hottest years in Perth.

2.2. Blood Analysis

Overnight fasted venous blood was collected at PathWest, Royal Perth Hospital for measurement of insulin, triacylglycerol (TAG), total cholesterol, and lipid fractions. Serum 25(OH)D was measured with a kit from Immunodiagnostic Systems, Boldon, UK. The kit quantifies all hydroxylated forms of vitamin D. The 25(OH)D kit had a sensitivity of 5 nmol/L with an intra-assay CV of 5.3%–6.7% and inter-assay CV of 4.6%–8.7%. Parathyroid hormone (PTH) was measured using a kit from Immutopics Inc., San Clemente, CA, USA. The PTH kit had a detection limit of 13 pg/mL, with intra-assay CV of 3%–5% and inter-assay CV of 4.7%–7.4%.

Markers of systemic inflammation and endothelial activation, e.g., hsCRP, TNF-α, HGF, P-selectin, sVCAM, sICAM, and leptin, were measured at the School of Public Health, Curtin University using a Human Adipokine Magnetic Bead Panel 2 (HADK2MAG-61K) and Human Cardiovascular Disease Magnetic Bead Panel 2 (HCVD2MAG-67K) and were run on a MAGPIX system (Merck Millipore, Luminex, Austin, TX, USA). Intra-assay CVs ranged from 3% for TNF-α to 5% for leptin, and inter-assay CVs ranged from 11% for insulin to 19% for TNF-α.

2.3. Statistical Analysis

The main outcome variables of this study were total cholesterol (TC), LDL-cholesterol, high density lipoprotein (HDL)-cholesterol, TAG, D-Dimer, sICAM, MPO, P-selectin, sVCAM, leptin, TNF, CRP, and HGF. The primary variable of interest was 25(OH)D, which was categorized into tertiles (T1 = highest, T2 = middle, T3 = lowest). Normality was assessed for all outcome variables and natural logarithm (or square root) transformation was applied if severe skewness was observed. One-way ANOVA was used to compare the differences between the tertiles for characteristics of subjects. Multivariate ANCOVA (MANCOVA) involved in multivariate general linear model (GLM) was used to test for differences in the main outcomes between tertiles of 25(OH)D, controlling initially for age, gender, BMI, McAuley's index, season, medications, and PTH. In the regression modelling, a backward elimination approach was then applied and variables that did not contribute to the model were removed at a 5% significance level. The final parsimonious model retained age, gender, BMI, and McAuley's index as significant confounders. Where overall significant effects were obtained, individual category comparisons were carried out separately for T1 and T2 against the lowest vitamin D tertile (T3), as a reference. McAuley's index and QUICKI were strongly related in our sample ($r = 0.843$; $p = 0.001$). We preferred McAuley's index since it was more strongly related to lipid endpoints in this study as compared to QUICKI (data not shown). Importantly, other authors have also endorsed its usefulness in reflecting aspects of insulin sensitivity in different population groups when compared to the gold standard clamp technique [17–19]. All analyses were performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp. Released 2013, Armonk, NY, USA) [20]. A significant difference was inferred when p values were less than 0.05.

3. Results

Eighty-three participants with complete data entered the final analysis. General characteristics of these volunteers are provided in Table 1.

Table 1. General characteristics of the study participants across tertiles of vitamin D status.

Variable	Tertile 1 ($N = 25$)	Tertile 2 ($N = 29$)	Tertile 3 ($N = 29$)	p Value
25(OH)D (nmol/L)	97.00 (83.00–202.60)	74.50 (66.00–83.00)	56.8 (26.34–65.42)	
Age (years)	65.85 (1.44)	65.12 (1.44)	64.96 (1.44)	0.720
Gender (M/F)	13 (39.4)/20(60.6)	11 (33.3)/22 (66.7)	20 (57.1)/15 (42.9)	0.119
Weight (kg)	81.38 (2.60)	78.27 (2.42)	76.34 (2.42)	0.945
BMI (kg/m^2)	28.18 (0.91)	28.45 (0.84)	27.62 (0.84)	0.609
SBP (mmHg)	148.12 (4.26)	143.00 (3.95)	142.69 (3.95)	0.734
DBP (mmHg)	84.52 (2.07)	80.14 (1.93)	81.41 (1.93)	0.387
PTH (pmol/L)	7.7 (13.88)	4.7 (2.74)	6.2 (4.81)	0.421
McAuley's index	8.3 (2.21)	8.3 (2.26)	8.3 (2.17)	0.999

Data are mean (SD) if continuous, except 25(OH)D [25 hydroxy vitamin D] which is median (range). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone.

3.1. 25(OH)D and Lipids Profiles

Based on the multivariate regression model, significant differences were found across 25(OH)D tertiles in TC (T1 < T3, $p = 0.003$; T2 < T3, $p = 0.001$) and LDL-C (T1 < T3, $p = 0.005$; T2 < T3, $p = 0.001$). HDL-C was not significantly different amongst the tertiles (Table 2). While a difference in TAG (T2 < T3,

$p = 0.04$) was noted, the MANOVA only showed an overall trend ($p = 0.082$) for the effect of 25(OH)D on TAG (Table 2).

Table 2. Vitamin D status and lipids profiles in older Australians.

Marker	Tertile 1 ($N = 25$)	Tertile 2 ($N = 29$)	Tertile 3 ($N = 29$)
25(OH)D (nmol/L)	97.0 (83.00–202.6)	74.5 (66.00–83.0)	56.8 (26.34–65.42)
TC (mmol/L)	4.87 ** (4.18–5.83)	4.91 † (3.92–5.85)	5.91 (4.52–6.48)
LDL-C (mmol/L)	2.80 ** (2.31–3.69)	2.79 † (2.16–3.55)	3.64 (2.60–4.09)
TAG (mmol/L) [1]	1.16 (0.46–2.60)	1.13 * (0.40–2.57)	1.37 (0.52 –2.50)
HDL-C (mmol/L)	1.30 (0.98–1.86)	1.43 (1.05–1.82)	1.45 (1.09–1.86)

Data are predicted median (range) from MANCOVA model that initially adjusted for age, gender, BMI, McAuley's index, season, medications, and PTH, but season, medications, and PTH were excluded in the final model by a backward elimination procedure. [1] Overall MANOVA, $p = 0.082$ for triacylglycerol (TAG), but $p < 0.05$ for total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C). High density lipoprotein cholesterol (HDL-C) was non-significant. Post-hoc tests * $p < 0.05$; ** $p < 0.005$; † $p < 0.001$; vs. Tertile 3.

3.2. Systemic Inflammatory Biomarkers and Endothelial Cells Activation Molecules

The multivariate regression model also revealed significant differences across 25(OH)D tertiles in HGF with T1 > T3 ($p = 0.009$, Table 3). While MANOVA showed that sVCAM in T1 is greater than that in T3 on average (T1 > T3 $p = 0.04$), the overall MANOVA effect of 25(OH)D on sVCAM only approached significance with a p value of 0.091 (Table 3). There were no other significant differences detected. Further adjustment for TNF-α and/or hsCRP did not modify any of these outcomes (data not shown).

Table 3. Systemic inflammatory and endothelial biomarkers across tertiles of vitamin D status.

Marker	Tertile 1 ($N = 25$)	Tertile 2 ($N = 29$)	Tertile 3 ($N = 29$)
25(OH)D (nmol/L)	97.0 (83.0–202.6)	74.5 (66.0–83.0)	56.8 (26.3–65.4)
TNF–α (pg/mL)	5.40 (3.80–9.12)	4.40 (3.10–6.00)	4.10 (3.10–6.46)
CRP (ng/L)	2134.50 (933.00–9120.00)	2079.00 (891.00–4467.00)	2359.00 (1122.0–9550.0)
Leptin (pg/mL)	7952 (4074–79433)	11871.4 (3020–40738)	8323.40 (2692–51286)
D–dimer (pg/mL)	2538 (1318–3162)	2258 (1513–3548)	1958 (1122–3630)
sICAM-1 (pg/mL)	126.00 (109.6–144.5)	114.40 (95.5–141)	132.77 (117.5–158.5)
MPO (pg/mL)	358.00 (302.00–447.00)	309.00 (219.00–417.00)	399.00 (288.40–489.80)
P-selectin (pg/mL)	130.00 (115.00–186.00)	141.00 (100.00–199.50)	159.00 (104.70–195.00)
sVCAM-1 (pg/mL) [1]	877.00 * (708.0–955.00)	705.80 (616.50–832.00)	703.00 (602.60–891.30)
HGF (pg/mL)	512.50 ** (355.00–633.50)	351.60 (263.00–509.00)	318.50 (219.00–426.80)

Data are predicted median (range) from MANCOVA model that initially adjusted for age, gender, BMI, McAuley's index, season, medications and PTH, but season, medications and PTH were excluded in the final model by a backward elimination procedure. [1] Overall MANOVA, $p = 0.091$ for sVCAM but $p < 0.05$ for HGF. Post-hoc tests * $p < 0.05$; ** $p < 0.01$ vs. Tertile 3. TNF-α, tumor necrosis factor-alpha; CRP, C reactive protein; sICAM, soluble intracellular adhesion molecule; MPO, myeloperoxidase; sVCAM, soluble vascular cell adhesion molecule; HGF, hepatocyte growth factor.

4. Discussion

Cardiovascular disease (CVD) is a significant contributor to the adverse health profile of Western Australians [21] and accounts for much of the State's health expenditure. Besides traditional serum lipid profiles and systemic inflammatory markers, endothelial dysfunction may underscore CVD, and measurement of endothelial cell activation could be important in determining this risk. A working model would hence place elevated TNF-α and hsCRP as indicators of systemic inflammation, and the pro-atherogenic state that occurs with dyslipidaemia would engender endothelial cell dysfunction and increased endothelial cell activation [22]. The latter would manifest as increased circulating levels of a variety of markers, including soluble vascular adhesion molecules, the selectins, pro-atherogenic

D-dimer and myeloperoxidase (MPO). Adequate 25(OH)D would then offset systemic inflammation, while potentially returning the endothelium to its quiescent state [6].

The majority of our older participants had a sufficient vitamin D status (25(OH)D > 50 nmol/L) as judged by current criteria [23,24]. This was not due to vitamin D supplement use, but could reflect the higher than normal annual temperature that year in Perth, where average annual temperatures of 25–26 °C were common on the coastal plain. We observed that higher tertiles of 25(OH)D were found to be associated with lower TC and LDL-C. Such data would argue that 25(OH)D is potentially protective of CVD risk even when vitamin D status is well above the recommended level (Table 2). There are now several studies that have examined the effect of vitamin D on lipid endpoints. Cross-sectional studies have indicated inverse associations between 25(OH)D and TAG [25] and a direct positive relationship with HDL-C [26]. Zimmerman et al. (2011) systematically reviewed the literature and concluded that cross-sectional studies favored the finding of a lower TAG, especially in studies where participants started from a higher TAG concentration [27]. Similarly, based on a very large dataset from one laboratory, Lupton et al. (2015) observed a significant inverse relationship between higher 25(OH)D and all circulating lipid markers [28]. The results of a meta-analysis of RCTs only partially confirmed these observational outcomes. While Chaloumas et al. (2014) found no impact of vitamin D supplementation on lipid markers [29], Manousopoulou et al. (2015) found a significant decrease in TAG [30]. In contrast, the latter review also reported a small increase in LDL-C with vitamin D [30]. Clearly, RCTs do not support a beneficial effect, while reviews of cross-sectional studies all show a decrease in TAG in particular. Hence, while our results are suggestive of a beneficial effect, a causal link between these observations requires further study for clarification [31].

Vitamin D could have a beneficial effect on endothelial function through a variety of effects including lowering systemic inflammation through reductions in TNF-α and hsCRP [32–35], and in decreasing endothelial cell activation as judged by lower sICAM-1 and sVCAM-1 [34,35]. Vitamin D may also increase plasma leptin, which could be vasodilatory to the endothelium [36,37]. Furthermore, low 25(OH)D levels leads to dysregulation in neutrophil activity leading to increased MPO levels [38]. On the other hand, 25(OH)D shows no correlation with P-selectin levels in the blood [32,34]. Endothelial cell activation molecules are indications of arterial health and function. These molecules—D-dimer, sICAM-1, sVCAM-1, MPO, P-selectin, and HGF—are amongst a host of others that have been used to study endothelial cell functioning [6,11,13]. While each molecule has a different role in the endothelium, in general they all show an increase with endothelial inflammation [39–47]. Contrary to our hypothesis, we found little significant evidence to support an association of 25(OH)D with most markers of endothelial cell activation that were measured (Table 3). In fact, with one marker, we observed a trend for the reverse; that those in the highest tertile of 25(OH)D had significantly higher sVCAM relative to the reference group (Table 3). However, it must be noted that the overall effect was marginally significant (Table 3). These outcomes did not change with further adjustment for inflammatory markers, TNF-α or hsCRP.

sVCAM-1 is expected to reflect membrane bound VCAM, which is involved in leukocyte migration, an early step in atherosclerosis. Although non-significant, the data may reflect the potential deleterious effects of very high levels of 25(OH)D. Interestingly, we also found that at these circulating levels of 25(OH)D (Tertile 3), HGF increased significantly more than in the reference group (Table 3). HGF has a major role in the endothelium, and is a potent angiogenic factor. It has the added benefit of preventing increased vascular permeability and leukocyte adhesion that is observed with another growth factor, vascular endothelial growth factor (VGEF). In fact, combination therapy of VGEF and HGF has been proposed for CVD [48]. It is possible that the increase in HGF at the highest tertile is a compensatory/adaptive response to counter deleterious effects of sVCAM.

5. Limitations

The cross-sectional nature of the study limits generalization and does not offer causation. Since this population group had relatively good vitamin D status and our sample was relatively small, we were

constrained in detecting potential differences in those with very poor vitamin status. Furthermore, as measures of habitual physical activity were unavailable, we could not control for its effect on CVD risk markers. As a fat-soluble vitamin, the potential deleterious effects of too high a status may also need consideration. While there is some evidence that 25(OH)D status and CVD mortality may be represented by a non-linear, reverse J shaped association [49], emerging long term data do not support this view [50].

6. Conclusions & Future Directions

Higher vitamin D status was associated with lower circulating lipid levels, namely lower total and LDL-cholesterol, and potentially lower TAG. In contrast, a higher circulating HGF may have a protective role in the endothelium. Future randomized controlled trials on a larger sample of patients with initial low vitamin D status would confirm whether vitamin D has a causal relationship with endothelial dysfunction. In this regard, a recent 4-month RCT on those at risk of type 2 diabetes found a significant improvement in arterial stiffness following both vitamin D_2 and vitamin D_3 supplementation [51]. Such data emphasize the potential impact of the long term outcomes of the VITAL trial [52].

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References

1. Australian, Bureau of Statistics 2012. Available online: http://www.abs.gov.au/ausstats/abs@.nsf/Lookup/4364.0.55.006Chapter2002011-12 (accessed on 19 April 2016).
2. Nowson, C.A.; McGrath, J.J. Vitamin D and health in adults in Australia and New Zealand: A position statement. *Med. J. Aust.* **2012**, *196*, 686–687. [CrossRef] [PubMed]
3. Joshi, D.; Center, J.R. Vitamin D deficiency in adults. *Aust. Prescr.* **2010**, *33*, 103–106. [CrossRef]
4. Nozza, J.M.; Rodda, C.P. Vitamin D deficiency in mothers of infants with rickets. *Med. J. Aust.* **2001**, *175*, 253. [PubMed]
5. Osteoporosis, Australia. Available online: http://www.osteoporosis.org.au/sites/default/files/files/Vit%20D%20Position%20Statement%2010%202013%20V2.pdf (accessed on 18 June 2015).
6. Dalan, R.; Liew, H. Vitamin D and the endothelium: Basic, translational and clinical research updates. *IJC Metab. Endoc.* **2014**, *4*, 4–17. [CrossRef]
7. Wang, L.; Song, Y. Circulating 25-hydroxy-vitamin D and risk of cardiovascular disease a meta-analysis of prospective studies. *Circ. Cardiovasc. Qual. Outcomes* **2012**, *5*, 819–829. [CrossRef] [PubMed]
8. Meigs, J.B.; Hu, F.B. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. *JAMA* **2004**, *291*, 1978–1986. [CrossRef] [PubMed]
9. Hewison, M. An update on vitamin D and human immunity. *Clin. Endocrinol.* **2012**, *76*, 315–325. [CrossRef] [PubMed]
10. Prietl, B.; Treiber, G. Vitamin D and immune function. *Nutrients* **2013**, *5*, 2502–2521. [CrossRef] [PubMed]
11. Calder, P.C.; Ahluwalia, N. A consideration of biomarkers to be used for evaluation of inflammation in human nutritional studies. *Br. J. Nutr.* **2013**, *109*, S1–S34. [CrossRef] [PubMed]
12. Bellia, A.; Garcovich, C. Serum 25-hydroxyvitamin D levels are inversely associated with systemic inflammation in severe obese subjects. *Intern. Emerg. Med.* **2013**, *8*, 33–40. [CrossRef] [PubMed]
13. Alyami, A.; Soares, M.J. Vitamin D & endothelial function. *Indian J. Med. Res.* **2014**, *140*, 483. [PubMed]

14. Ridker, P.M.; Hennekens, C.H. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N. Engl. J. Med.* **2000**, *342*, 836–843. [CrossRef] [PubMed]
15. Lavie, C.J.; Lee, J.H.; Milani, R.V. Vitamin D and Cardiovascular Disease. Will, It, Live, Up to its Hype? *J. Am. Coll. Cardiol.* **2011**, *58*, 1547–1556. [CrossRef] [PubMed]
16. Lam, V.; Albrecht, M.A. Serum 25-hydroxy Vitamin D is associated with reduced verbal episodic memory in healthy, middle-aged and older adults. *Eur. J. Nutr.* **2016**, *55*, 1503–1513. [CrossRef] [PubMed]
17. Ascaso, J.F.; Pardo, S. Diagnosing insulin resistance by simple quantitative methods in subjects with normal glucose metabolism. *Diabetes Care* **2003**, *26*, 3320–3325. [CrossRef] [PubMed]
18. Lorenzo, C.; Haffner, S.M. Relation of direct and surrogate measures of insulin resistance to cardiovascular risk factors in nondiabetic, Finnish offspring of type 2 diabetic individuals. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 5082–5090. [CrossRef] [PubMed]
19. Venkatesan, P.; Tiwari, A. Surrogate measures of insulin sensitivity when compared to euglycemic hyperinsulinemic clamp studies in, Asian, Indian men without diabetes. *J. Diabetes Complicat.* **2016**, *30*, 287–291. [CrossRef] [PubMed]
20. Field, A. *Discovering Statistics Using IBM SPSS Statistics*, 4th ed.; Sage: London, UK, 2013; pp. 249–257.
21. Sarink, D.; Nedkoff, L. Projected age-and sex-specific prevalence of cardiovascular diseases in, Western, Australian adults from 2005 to 2045. *Eur. J. Prev. Cardiol.* **2016**, *23*, 23–32. [CrossRef] [PubMed]
22. Szmitko, P.E.; Wang, C.H. New markers of inflammation and endothelial cell activation part I. *Circulation* **2003**, *108*, 1917–1923. [CrossRef] [PubMed]
23. Holick, M.F. Vitamin D deficiency. *N. Eng. J. Med.* **2007**, *357*, 266–281. [CrossRef] [PubMed]
24. Lips, P.; Bouillon, R. Review article: Reducing fracture risk with calcium and vitamin D. *Clin. Endocrinol.* **2010**, *73*, 277–285. [CrossRef] [PubMed]
25. Jorde, R.; Figenschau, Y. High serum 25-hydroxyvitamin D concentrations are associated with a favorable serum lipid profile. *Eur. J. Clin. Nutr.* **2010**, *64*, 1457–1464. [CrossRef] [PubMed]
26. Jorde, R.; Grimnes, G. Vitamin D and metabolic health with special reference to the effect of vitamin D on serum lipids. *Prog. Lipid Res.* **2011**, *50*, 303–312. [CrossRef] [PubMed]
27. Zittermann, A.F.; Gummert, J. The role of vitamin D in dyslipidemia and cardiovascular disease. *Curr. Pharm. Des.* **2011**, *17*, 933–942. [CrossRef] [PubMed]
28. Lupton, J.R.; Faridi, K.F. Deficient serum 25-hydroxyvitamin D is associated with an atherogenic lipid profile: The Very Large Database of Lipids (VLDL-3) study. *J. Clin. Lipidol.* **2016**, *10*, 72–81. [CrossRef] [PubMed]
29. Challoumas, D. Vitamin D supplementation and lipid profile: What does the best available evidence show? *Atherosclerosis* **2014**, *235*, 130–139. [CrossRef] [PubMed]
30. Manousopoulou, A.; Al-Daghri, N.M. Vitamin D and cardiovascular risk among adults with obesity: A systematic review and meta-analysis. *Eur. J. Clin. Invest.* **2015**, *45*, 1113–1126. [CrossRef] [PubMed]
31. Pannu, P.K.; Calton, E.K.; Soares, M.J. Chapter Two-Calcium and Vitamin D in Obesity and Related Chronic Disease. *Adv. Food. Nutr. Res.* **2016**, *77*, 57–100. [PubMed]
32. Judd, S.E.; Tangpricha, V. Vitamin D therapy and cardiovascular health. *Curr. Hypertens. Rep.* **2011**, *13*, 187–191. [CrossRef] [PubMed]
33. Wamberg, L.; Kampmann, U. Effects of vitamin D supplementation on body fat accumulation, inflammation and metabolic risk factors in obese adults with low vitamin D levels—Results from a randomized trial. *Eur. J. Intern. Med.* **2013**, *24*, 644–649. [CrossRef] [PubMed]
34. Martinesi, M.; Treves, C. Vitamin D derivatives induce apoptosis and downregulate, ICAM-1 levels in peripheral blood mononuclear cells of inflammatory bowel disease patients. *Inflamm. Bowel Dis.* **2008**, *14*, 597–604. [CrossRef] [PubMed]
35. Van den Bemd, G.J.; Pols, H.A. Anti-tumor effects of 1,25-dihydroxyvitamin D_3 and vitamin D analogs. *Curr. Pharm. Des.* **2000**, *6*, 717–732. [CrossRef] [PubMed]
36. Bełtowski, J.; Jamroz-Wiśniewska, A. Modulation of H_2S metabolism by statins: A new aspect of cardiovascular pharmacology. *Antioxid. Redox Signal.* **2012**, *17*, 81–94. [CrossRef] [PubMed]
37. Jamroz-Wiśniewska, A.; Gertler, A. Leptin-induced endothelium-dependent vasorelaxation of peripheral arteries in lean and obese rats: Role of nitric oxide and hydrogen sulfide. *PLoS ONE* **2014**, *9*, e86744. [CrossRef] [PubMed]

38. Handono, K.; Hasanah, D. Low serum level of vitamin D is associated with increased number of low-density granulocytes (LDGs) and neutrophil extracellular traps (NETs) formation in patients with systemic lupus erythematosus. *GJBAHS* **2013**, *2*, 90–95.
39. Carr, J.M.; McKinney, M. Diagnosis of disseminated intravascular coagulation. Role of D-dimer. *Am. J. Clin. Pathol.* **1989**, *91*, 280–287. [CrossRef] [PubMed]
40. Witkowska, A.M. Soluble ICAM-1: A marker of vascular inflammation and lifestyle. *Cytokine* **2005**, *31*, 127–134. [CrossRef] [PubMed]
41. Kim, I.; Moon, S.O. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin through nuclear factor-κB activation in endothelial cells. *J. Biol. Chem.* **2001**, *276*, 7614–7620. [CrossRef] [PubMed]
42. Arnhold, J. Properties, functions and secretion of human myeloperoxidase. *Biochemistry (Mosc)* **2004**, *69*, 4–9. [CrossRef] [PubMed]
43. Pan, J.; Xia, L. Comparison of promoters for the murine and human P-selectin genes suggests species-specific and conserved mechanisms for transcriptional regulation in endothelial cells. *J. Biol. Chem.* **1998**, *273*, 10058–10067. [CrossRef] [PubMed]
44. Stocker, C.J.; Sugars, K.L. TNF-α, IL-4, and, IFN-γ regulate differential expression of, P-and E-selectin expression by porcine aortic endothelial cells. *J. Immunol.* **2000**, *164*, 3309–3315. [CrossRef] [PubMed]
45. Fantuzzi, G.; Faggioni, R. Leptin in the regulation of immunity inflammation and hematopoiesis. *J. Leukoc. Biol.* **2000**, *68*, 437–446. [PubMed]
46. Xin, X.; Yang, S. Hepatocyte growth factor enhances vascular endothelial growth factor-induced, angiogenesis in vitro and in vivo. *Am. J. Pathol.* **2001**, *158*, 1111–1120. [CrossRef]
47. Bradley, J. TNF-mediated inflammatory disease. *J. Pathol.* **2008**, *214*, 149–160. [CrossRef] [PubMed]
48. Min, J.K.; Lee, Y.M. Hepatocyte growth factor suppresses vascular endothelial growth factor-induced expression of endothelial, ICAM-1 and, VCAM-1 by inhibiting the nuclear factor-κB pathway. *Circ. Res.* **2005**, *96*, 300–307. [CrossRef] [PubMed]
49. Durup, D.; Jørgensen, H.L. A reverse J-shaped association, between serum 25-hydroxyvitamin D and cardiovascular disease mortality: The Copd study. *J. Clin. Endocrinol. Metab.* **2015**, *100*, 2339–2346. [CrossRef] [PubMed]
50. Daraghmeh, A.H.; Bertoia, M.L.; Mazen, O.; Al-Qadi, M.O.; Abdulbaki, A.M. Evidence for the vitamin D hypothesis: The N.HANES III extended mortality follow-up. *Atherosclerosis* **2016**. [CrossRef]
51. Forouhi, N.G.; Menon, R.K. Effects of vitamin D2 or D3 supplementation on glycaemic control and cardiometabolic risk among people at risk of type 2 diabetes: Results of a randomized double-blind placebo-controlled trial. *Diabetes Obes. Metab.* **2016**, *18*, 392–400. [CrossRef] [PubMed]
52. LeBoff, M.S.; Yue, A.Y. VITAL-Bone, Health: Rationale and design of two ancillary studies evaluating the effects of vitamin D and/or omega-3 fatty acid supplements on incident fractures and bone health outcomes in the, VITamin D and, OmegA-3 Trial. (VITAL). *Contemp. Clin. Trials* **2015**, *41*, 259–268. [CrossRef] [PubMed]

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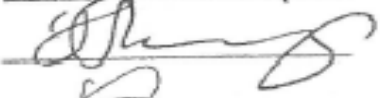
To Whom It May Concern

I, **Ali Mahdi Alyami**, contributed *(**designed the table and wrote the first draft**)* to the paper/publication entitled (**Alyami, A. M., Lam, V., Soares, M. J., Zhao, Y., Sherriff, J. L., Mamo, J. C., James, A.P. & Coombes, F. (2016). The association of vitamin D status with dyslipidaemia and biomarkers of endothelial cell activation in older Australians. Nutrients, 8(8), 457.**)

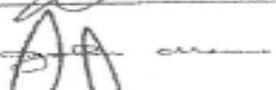
Candidate: Ali Alyami **Signature:** 

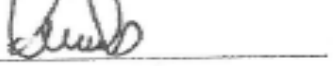
I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Co-Author 1: Mario Soares **Signature:** 

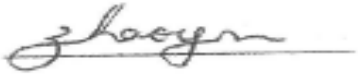
Co-Author 2: Jillian Sherriff **Signature:** 

Co-Author 3: Virginie Lam **Signature:** 

Co-Author 4: John Mamo **Signature:** 

Co-Author 5: Fiona Coombes **Signature:** 

Co-Author 6: Anthony P. James **Signature:** 

Co-Author 7: Yun Zhao **Signature:** 

Additional details about the original study for chapter 4:

Our study sample was randomly drawn from an original study with a total of 285 healthy participants (free from major surgery in the last six months, haemophilia, HIV, and cancer) living in Perth Western Australia. The original study had a different purpose, and did not collect physical activity data.

We acknowledge that the participants in our sample had relatively good 25(OH)D levels, and hence do not fully represent all the variations in levels in the Australian population. Unfortunately, the lack of physical activity data, and time spent outside could not be controlled for in our analysis. This information would have further confirmed the putative effects on lipids profile, 25(OH)D levels, and CVD risk markers. Furthermore, the potential confounding factors are not controlled for in cross-sectional studies as they would be in RCTs. In the statistical analysis, we controlled for a variety of the confounding factors, but it is possible that some of the confounding factors were not accounted for.

Manipulation of the independent variable (25(OH)D) and regular serial measurements of the dependent variables (lipids profile, endothelial cell activation molecules) in two or more groups over a period of time would have formed a stronger case for the effect of the 25(OH)D on endothelial dysfunction. However, due to limited funds, this could not be achieved.

Chapter 5:

Variations in endothelial function and insulin sensitivity following oral glucose: the impact of vitamin D status and ethnicity.

Chapter 5: Variations in endothelial function and insulin sensitivity following oral glucose: the impact of vitamin D status and ethnicity.

Introduction

T2DM and CVD are becoming increasingly prevalent around the globe. They share a multifactorial aetiology; progressively sedentary lifestyles and increasing rates of obesity are accepted as major contributors to this disturbing trend. Collectively, these two diseases represent the greatest disease burden around the world and are responsible for greater than half of global mortality (8, 9).

T2DM and CVD represent unique pathological states wherein it seems that inflammation and vascular pathology play more interlinked and fundamental roles than previously thought. T2DM develops insidiously over a period of years, with the individual progressing from a pre-diabetic to a diabetic state in the absence of corrective lifestyle or medical intervention. Once T2DM is established, it usually represents a lifelong health condition, and often leads to cardiovascular compromise (13). Therefore, early detection and a focus on prevention are paramount to reducing T2DM onset, progression to CVD and CVD-related mortality rates (14).

Epidemiology of T2DM and CVD and Vitamin D deficiency

In Australia, 7.2% of the population, or 1,086,000 people, were diagnosed with T2DM in 2010 and 1,068,000 adults were diagnosed in 2016 (12, 257). It is predicted that the prevalence of T2DM in Australia will rise to 8.4%, or 1,503 million people, by 2030 (257). In contrast, in the Kingdom of Saudi Arabia (KSA), 10.5% of the population were diagnosed with T2DM in 2009; this figure rose to 13.6% or 2,065,000 people in 2010 (257, 258). Furthermore, the prevalence of T2DM is expected to increase to 17.0% or 4,183,000 people in 2030 (257). A more recent study in the KSA found that 12.9% of men and 11.4% of women had T2DM in 2016, representing an overall prevalence of 12.1% (259), with a further 9.4% of men and 8.6% of women diagnosed with pre-diabetes (259). In Australia, a recent self-reported survey found that 22% of both men and women were diagnosed with one or more CVD in 2014-15 (10). In the KSA, CVD was responsible for 46% of deaths in 2014 (260). Therefore, there is an urgent need for prevention of these diseases.

Vitamin D deficiency (25(OH)D < 50 nmol/L) is observed in most countries around the world (2). Levels lower than 25 nmol/L have been observed in the Middle East (2). The associated risk factors for vitamin D deficiency include advanced age, darker skin colour, female gender, obesity, inadequate sunlight exposure, seasonal variations, poor dietary availability of vitamin D and vitamin D fortification (6). A study conducted in three locations around Australia highlighted the differences

in vitamin D deficiency between seasons and latitude. In winter and spring, prevalence of vitamin D deficiency (25(OH)D <50 nmol/L) were found to be 40.5% in southeast Queensland, 37.4% in Geelong, Victoria and 67.3% in Tasmania (98). Meanwhile, in summer and autumn, subsets of the same populations had levels of 25(OH)D <25 nmol/L, namely 7.1 % in southeast Queensland, 7.9% in Geelong and 13.0% in Tasmania (98). Overall prevalence of vitamin D deficiency (25(OH)D <50 nmol/L) was 18% in southeast Queensland, 10% in Geelong and around 32% in Tasmania (98). Total vitamin D deficiency (25(OH)D <50 nmol/L) is present in 23% of Australian adults, with a greater prevalence in older Australians (4, 5). For example, a moderate to severe vitamin D deficiency (25(OH)D <30 nmol/L) was found in 68% of men and 86% of women living in an aged-care facility in Sydney (99), in 67% of elderly patients (mean age 81 years) admitted to hospital with hip fractures in Tasmania (100) and in 17% of elderly Tasmanian people (mean age 79 years) living within the communities (101).

The Middle East has high ambient sunshine that would render appropriate levels of vitamin D synthesis easier than in other locations, however many areas have a cultural propensity towards covering the body which has led to high rates of vitamin D deficiency. For instance, a 2003 study of 299 participants showed that 83% of participants had 25(OH)D <25 nmol/L (147). Also, a 2012 cross-sectional study performed on healthy men found 25(OH)D levels <25 nmol/L in 48.9% of participants and levels between 25 nmol/L and 50 nmol/L in 38.8% (149). The prevalence in children was higher; in Riyadh KSA, a 2012 study of 331 children found that 83% had 25(OH)D levels of < 25 nmol/L and 16.8 % had 25(OH)D levels between 25 – 29.9 nmol/L (145). A later cohort study conducted in 2014 on 3,475 Saudi adults found that the mean 25(OH)D value was 35.5 nmol/L, with 36.1% of females and 48.8% of males displaying values of <25 nmol/L (148). Among adults in the KSA, a 2016 study showed that the mean 25(OH)D levels are also low at approximately 40 nmol/L among men and lower, at 31 nmol/L among women (146). A limitation of these studies is that they analysed 25(OH)D levels using radioimmunoassay (DiaSorin Liaison), which has now been superseded by LC-MS/MS (261).

The cut-off for vitamin D deficiency (25(OH)D < 50 nmol/L) was set predominately for the purpose of maintaining bone health (5). There is increasing discussion that a new vitamin D threshold has to be proposed to address health problems that are extraneous to bone health (5, 60).

Relationship between T2DM, CVD, insulin resistance and endothelial function:

Insulin resistance (IR) is one of the hallmark pathological features of T2DM. IR is the inability of body cells to maintain normal glucose and lipid homeostasis, leading to the need for higher levels of

insulin to maintain a normo-glycaemic state (27, 28). Risk factors for IR include genetic susceptibility, ageing, sedentary lifestyle and obesity (11). IR is strongly correlated with visceral obesity, hypertension, atherogenic dyslipidaemia and pro-thrombotic states (11). The presence of both IR and developing CVD is collectively referred to as a 'metabolic syndrome' (MetS) (11). Thus, MetS is an indicator of developing CVD as well as possible progression to T2DM (11) with IR being central to both CVD and T2DM, and therefore, to MetS (11). A body of evidence also implicates both IR and endothelial dysfunction (ED) in atherosclerosis and cardiovascular demise (29). This is not surprising considering the interdependent relationship between endothelial function and insulin secretion. Not only does ED impede the trans-capillary movement of insulin, restricting its access to target cells (29), but an impairment of insulin secretion alters the balance between vasodilators and vasoconstrictors and subsequently alters endothelial function (see endothelial dysfunction and its role in insulin resistance and metabolic syndrome section in the literature review chapter).

Furthermore, nitric oxide (NO) is a vasoactive gas produced by the endothelial cells of blood vessels to regulate various systems, especially local vessel diameter. Local NO concentrations provide a strong measure of endothelial function (45). ED leads to reduced nitric oxide (NO) dependent vascular activity, which is consistent with IR states (45). Endothelial-derived NO secretion is lower when ED is present, which links the progression of ED and IR (29, 55). Evidently, these variables are interrelated in ways that are deleterious to maintaining physiological cardiovascular and metabolic mechanisms.

Methods of assessing endothelial function

Endothelial function (EF) is usually evaluated by assessing the coronary or peripheral arterial responses to controlled stimuli (26). There are two ways of achieving this. The first way is to test endothelial-dependent vasodilation. The second way is to measure endothelial-independent vasodilation, which bypasses the endothelial cells and works on the smooth muscle to cause relaxation of the vascular wall (26). The gold standard technique uses both intracoronary agonist infusion and quantitative angiography, which involves the invasive procedure of artery catheterisation (26, 53) (see endothelial function testing section in the literature review chapter).

However, non-invasive techniques have gained credibility for research purposes. These include; flow-mediated dilation (FMD) measured by ultrasound (US) imaging (47, 54); the central augmentation index (AIx) can be measured directly using carotid arterial tonometry or indirectly by a generated transfer function (TF) technique, and the radial AIx can be measured by radial arterial

tonometry (160); pulse-wave velocity (PWV) is calculated from the time between two blood pressure wave forms in different arterial sites (162); and pulse contour analysis (PCA) is measured by generating a digital volume pulse (DVP) to calculate reflective index (RI) and stiffness index (SI) (55).

EF can be evaluated through the measurement of systemic inflammatory biomarkers such as C-reactive protein (CRP), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα). EF can also be assessed by the levels of endothelial cell activation molecules such as soluble vascular cellular adhesion molecule (sVCAM), soluble intracellular adhesion molecule (sICAM), P-selectin and E-selectin (26, 47). The relationships between these biomarkers and EF are discussed extensively in our previous review paper (26).

The present study utilises PCA as our research team has expertise with this method and it is a credible method for clinical evaluation of endothelial function. The method uses a pulse trace system to generate a DVP using finger photoplethysmography (55). Subsequently, the system calculates two outcomes: stiffness index (SI), which is the time from first peak to second peak (PPT) which has output as time (sec)/height (m) and reflective index (RI), which is a ratio of wave [A] amplitude to wave [B] amplitude (Figure 3) (55). SI evaluates larger arteries while RI assesses smaller vessel tone (55). A lower RI is indicative of vascular vasodilation and a higher RI is indicative of vasoconstriction, where RI is generally between 60% and 90% in healthy (253). Meanwhile, lower SI means less arterial stiffness with a value of 5 to 8 meters per second (m/s) in healthy people in their twenties and 6 to 10 m/s in healthy people in their sixties (262).

Objectives & hypothesis

This study was aimed at discerning the relationship between vitamin D status and EF measured by PCA among two ethnic groups: Australians and Middle Easterners. We hypothesised that people with vitamin D deficiency would have signs of endothelial dysfunction, indicated by higher RI and SI values, compared to the people with sufficient vitamin D levels.

Methods

Participant sampling

Radio and community advertising was used to recruit both Middle Eastern and Australian of European descent participants. Participants interested in the study were prompted to contact the lead researcher. Participants were subsequently sent study information and an initial screening form. Subjects were screening using our selection criteria: adults aged >18 years, lactose tolerant, stable

weight (± 2 kg) over the last six months, not suffering conditions involving the thyroid, liver, kidney or heart, or conditions such as osteoporosis, hypertension, gastrointestinal problems, not suffering illness or infection requiring antibiotic therapy, have not had gastrointestinal surgery, not on any medication or supplements (including vitamin D, fish oil or thiamin supplements) or any special or commercial dieting programs that may affect the body's metabolism, and not taking part in strenuous physical activities (subjects engaged in competitive sports or team games or running [4.3 min/km] or jogging [5.6 min/km] excluded) (243). If participants met the selection criteria they were contacted and provided with a time for an orientation appointment at our laboratory. At this appointment, participants were provided with the pre-test meal, used to reduce dietary variability.

IR was measured following a conventional 75-g oral glucose tolerance test (OGTT) and calculation of fasting and postprandial indices. For measuring EF, Digital Volume Pulse Trace (PT-2000) was used in both fasted and fed states as illustrated in Figure 7. All participants provided written informed consent before participating in the study. These studies were approved by the Human Research Ethics Committee at Curtin University, Perth, Western Australia (Approval number: HR72/2013 and HR 103/2012).

Study Protocol

Before coming to the Health Centre, participants fasted overnight following a low sodium and low fat meal that was provided at no cost. Participants continued to refrain from engaging in any strenuous physical activity in the evening and morning prior to attending the laboratory. On arrival, participants emptied their bladders, then were weighed and had anthropometry measured. After this, they were rested in the supine position for 30 min before an oral glucose load of 75 g was administered to each subject. Thereafter, serial measurements of blood pressure and endothelial function were taken over a two-hour period (Figure 7). During these two hours, each person was connected to the PT-2000 device and his/her arterial stiffness was measured every 30 minutes. Venous blood samples were drawn at fasting and 2 hours after OGTT, for measurements of insulin, glucose concentrations, vitamin D, inflammation and endothelial biomarkers.

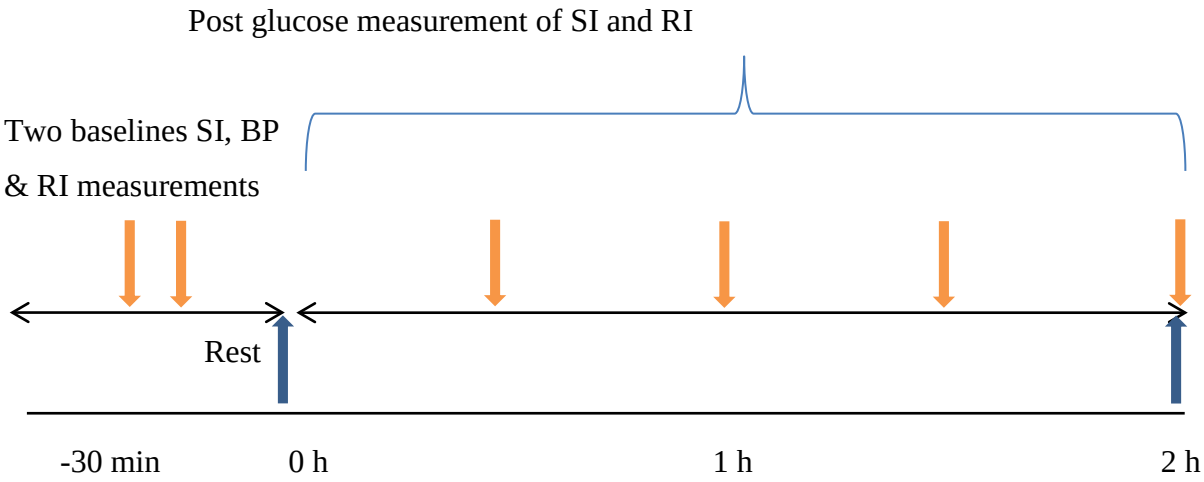


Figure 7: Study two protocol.

0 h corresponds with the beginning of blood pressure and endothelial function assessments. Prior, subject rested for half an hour, then endothelial function was measured using PCA for RI and SI, thereafter blood samples for glucose, insulin and vitamin D quantification were drawn. At 0 h, 75 g glucose load was administered to each subject (per oral). From point zero onwards, endothelial function was intermittently measured in duplicate at 30-minute intervals for the duration of two hours. At 2 h, a second round of blood samples was collected for glucose and insulin quantification. Note: Each measurement of PCA uses at least 10 wave forms to calculate SI and RI, so 20 wave forms were recorded for each time point.

Analysis of study outcomes

Endothelial function was measured using the Pulse Contour Analysis (PCA) method. The main outcome in this analysis was RI. A decrease in RI indicates healthy endothelial function (Figure 8). This is due to the ability of healthy subjects to track the changes faster in small blood vessels in response to an endothelial-dependent stimulus (insulin as a response to glucose ingestion). Meanwhile, SI assesses large arteries where the changes occur over a comparatively longer timeframe than RI. The body composition and fat mass of all subjects was examined by dual energy x-Ray absorptiometry (DEXA) (Prodigy, Luna Corporation, USA) (263). The presence of MetS was determined following the latest guidelines (245). Fasting insulin sensitivity was calculated by McAuley's index (Mc_ISI), using fasting insulin and triglyceride concentrations (248). Fasting and postprandial changes in insulin sensitivity were monitored through the insulin sensitivity index at time zero (ISI_0) and time 120 min (ISI_120) using the equation of Sluiter et al (249). Blood analysis was conducted by the Royal Perth Hospital Pathology Department, Perth, Western Australia. A chemiluminescent immunoassay (Liaison, DiaSorin and Architect, Abbott) was used to measure 25(OH)D concentrations.

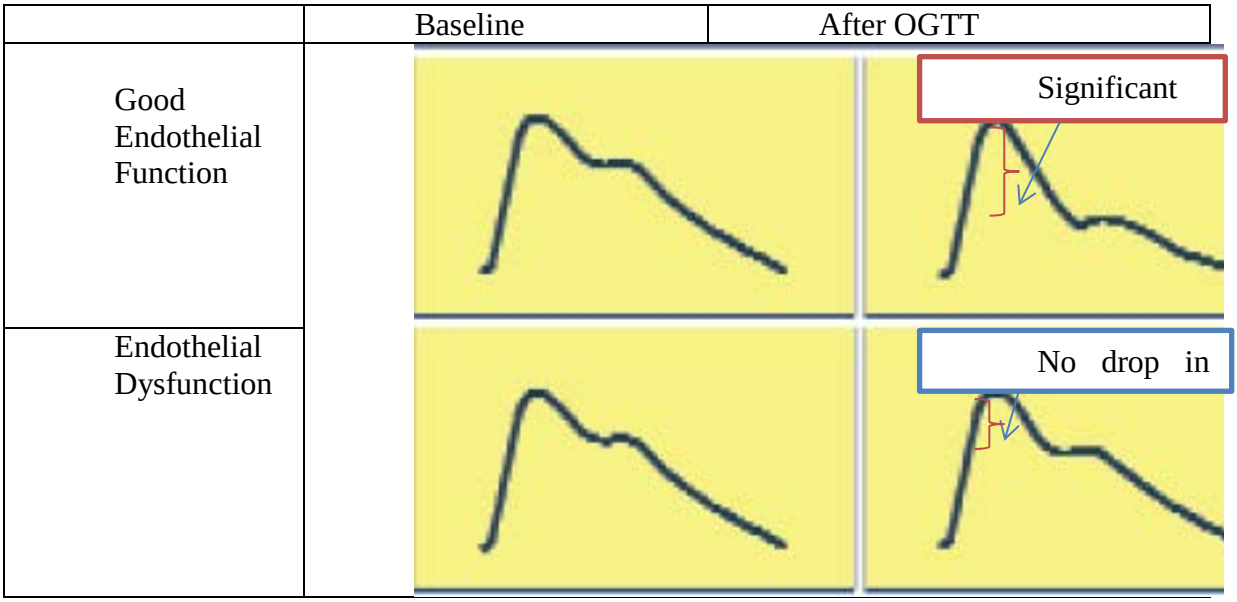


Figure 8: Expected endothelial function test (RI) and baseline outcomes during the 75 g OGTT.

As shown, a decrease in the RI is suggestive of appropriate endothelial functioning, while no decrease in RI in response to the glucose load is indicative of endothelial dysfunction.

Statistical Analysis

All continuous variables were assessed for normality prior to further statistical analysis. Descriptive parameters were summarised by mean and standard deviation values. The following were assessed in both groups using one-way analysis of variance (ANOVA): physical activity (METS/min/week), free fat mass (FFM), basal RI, basal SI, basal heart rate (HR), basal mean arterial pressure (MAP), fasting vitamin D, fasting ISI and post-prandial change in ISI. We calculated integrated area under the curve (IAUC) of SI (IAUC-SI), IAUC of RI (IAUC-RI), IAUC of HR (IAUC-HR) and IAUC of MAP (IAUC-MAP) to measure the changes in these EF measurements after glucose ingestion. Multivariate analysis of covariance (MANCOVA) was used to examine the role of ethnicity among the changes in IAUC-RI, IAUC-SI, IAUC-HR, IAUC-MAP, ISI, sICAM and monocyte chemoattractant protein 1 (MCP-1), after adjustment for age, FM, FFM, season, 25OHD physical activity, A/G ratio **.**

Results

A total of 38 participants were assessed in this study. Of these subjects, 21 were Australian men of European descent and 17 were Middle Eastern men having live in Australia for a period of greater than one year. These participants were assessed in two separate studies. Table 7 contains the demographic and baseline data of the participants. The mean ages among the study population were 52.4 years in Australian men and 28.9 years in Middle Eastern men. The mean fat mass across the study population were 32.3 kg in the Australian men group and 25.9 kg in the Middle Eastern men group. The fat free mass values (FFM) and android/gynoid ratios (A/G ratio) were higher in the Australian men with 64.6 kg and 1.3 compared to 51 kg and 1.1 in the Middle Eastern men, respectively. Also, the Australian men had higher BMI (30.7 kg/m^2) compared with the Middle Eastern men (26.4 kg/m^2). From a physical standpoint, the android/gynoid (A/G) ratio was used as a measure of android fat distribution using DEXA.

Table 7: General characteristics of the participants.

Variable	**Australian Men (N=21)**		**Middle Eastern Men (N=17)**		**P-value**
	Mean	SEM	Mean	SEM	
Age (years)	52.4	2.94	28.9	3.09	0.001
BMI (kg/m^2)	30.7	6.24	26.4	7.35	0.057
Fat Mass (kg)*	32.3	2.76	25.9	2.90	0.117
Fat Free Mass (kg)*	64.6	1.90	51.0	2.00	0.001
A/G ratio*	1.3	0.05	1.1	0.06	0.005

BMI: body mass index, A/G ratio: android/gynoid ratio. *measured by DEXA

After adjustment for age, fat mass, fat free mass, physical activity and A/G ratio, the physiological baseline values for 25(OH)D were higher among Australian men at 60.9 nmol/L and lowest among Middle Eastern men at 40.4 nmol/L but the comparison only trended towards significance (Table 8). Thus the Middle Eastern men were in the vitamin D deficiency category (<50 nmol/L). There were no statistically significant differences among endothelial function measurements including reflective index (RI) and stiffness index (SI). However, in Australian men, SI was slightly higher while RI was slightly lower. The average fasting heart rate (HR) was similar amongst participants of both ethnic backgrounds. Australian men had higher fasting glucose levels and mean arterial pressure (MAP) compared to Middle Eastern men. However, the fasting insulin sensitivity index (ISI) using Sluiter et al equation (249) was higher among the Middle Eastern. In the Middle Eastern men, the soluble

intracellular adhesion molecule (sICAM) was comparatively high. Fasting monocyte chemoattractant protein-1 (MCP-1) was higher in Australian men compared with Middle Eastern men.

Table 8: General characteristics of the participants after adjustment for age, fat mass, fat free mass, physical activity, seasonal variations and A/G ratio.

Variable	Australian Men (N=21)		Middle Eastern Men (N=17)		P value
	Mean	SEM	Mean	SEM	
Fasting 25(OH)D (nmol/L)	60.9	5.34	40.4	6.27	0.060
Fasting RI	68.4	3.67	69.8	4.31	0.850
Fasting SI	8.2	0.48	6.9	0.56	0.190
Fasting HR (bpm)	63.5	2.58	58.6	3.02	0.337
Fasting MAP	92.9	2.99	89.5	3.51	0.569
Fasting Glucose (mmol/L)	5.7	0.27	5.4	0.32	0.654
Fasting Insulin (IU/L)	8.8	1.68	8.0	1.97	0.811
Fasting ISI	14.5	3.10	21.1	3.64	0.288
Fasting MCP-1**	226.1	67.76	159.8	70.17	0.603
Fasting sICAM**	123.1	15.40	146.6	15.95	0.420

One way ANOVA for comparison between groups, A/G ratio: android/gynoid ratio, RI: reflective index, SI: stiffness index, HR: heart rate, MAP: mean arterial pressure, ISI: Insulin Sensitivity Index. Fasting insulin levels were measured using Sluiter equation for insulin sensitivity index. MCP-1: monocyte chemoattractant protein 1 and sICAM: soluble cell adhesion molecules. *Measured using dual energy x-ray absorptiometry (DEXA). ** MCP-1 and sICAM analysis includes only 18 Australian and 17 Middle Eastern men.

There were no significant differences among the two groups in fasting RI, SI, insulin, glucose, MCP-1 and sICAM after adjustment for age, free fat mass (FFM), fat mass (FM), physical activity using international physical activity questionnaire (IPAQ) and seasonal variations using MANCOVA (Table 8). Although not significant, ISI was slightly higher in Middle Eastern men compared to Australian men using the same adjustments.

In the postprandial state, MANCOVA was performed for the change in the means after adjustment for age, FFM, FM, physical activity (IPAQ) and seasonal variations. In each group, we detected a statistically significant decrease in the adjusted mean of postprandial ISI within the group (Table 9). The Middle Eastern men had a statistically significant decrease in the adjusted mean of ISI (p=0.021) compared to Australian men. However, there were no statistically significant differences among IAUC-SI, IAUC-HR, IAUC-MAP, MCP-1 and sICAM within or between the two groups.

Table 9. MANCOVA analysis of the absolute change 2 h following oral glucose among two ethnics groups.

Change in the variable after two hours	Australian Men		Middle Eastern Men		
	Mean	**SEM**	**Mean**	**SEM**	**P value**
IAUC-RI	-5.9	2.98	-0.4	3.49	0.349
IAUC-SI	-0.1	0.74	-0.7	0.9	0.668
IAUC-HR	2.0	2.46	5.9	2.89	0.429
IAUC-MAP	0.5	3.2	-0.7	3.8	0.854
MCP-1 pg/ml**	-5.1	13.20	-0.2	13.84	0.843
sICAM pg/ml**	-13.4	14.00	-35.5	14.68	0.400
Postprandial ISI (10,000/Io x Go)	-7.5*	3.13	-22.5*	3.67	0.021

All data adjusted for age, free fat mass, fat mass, physical activity, A/G ratio and seasonal variation. IAUC-RI for reflective index. IAUC-SI for stiffness index, IAUC-HR for heart rate, IAUC-MAP for mean arterial pressure, MCP-1: monocyte chemoattractant protein 1, sICAM: soluble intracellular adhesion molecule. *P <0.05 within each group. ** MCP-1 and sICAM analysis includes only 18 Australian and 17 Middle Eastern men.

After ingestion of 75-g glucose, Middle Eastern men demonstrated no decrease in the adjusted mean of IAUC-RI, which suggested a slight endothelial dysfunction. In contrast, the Australian men showed a substantial decrease in the change of the adjusted mean of the IAUC-RI which showed greater endothelial vasodilation. However, there were no significant differences in the change of the adjusted means of IAUC-SI, IAUC-HR and IAUC-MAP among the two ethnic groups.

Both groups showed a decline in the adjusted mean of the postprandial ISI, however the decline was significantly greater in the Middle Eastern men than the Australian men. There was no difference in the change of the adjusted mean of MCP-1, while there was a greater decline in the percentage-change of the mean sICAM in the Middle Eastern men compared to the Australian men.

Table 10. MANCOVA analysis of the absolute change 2 hr following oral glucose among two ethnics groups after further adjustment for 25(OH)D:

Change in the variable after two hours	Australian Men		Middle Eastern Men		
	Mean	**SEM**	**Mean**	**SEM**	**P value**
IAUC-RI	-5.2	3.18	-1.0	3.68	0.509
IAUC-SI	0.3	0.77	-1.1	0.89	0.383
IAUC-HR	1.9	2.65	6.0	3.07	0.446
IAUC-MAP	-0.2	3.44	0.1	3.98	0.959
MCP-1 pg/ml	-9.4	13.97	3.6	14.43	0.622
sICAM pg/ml	-16.6	14.93	-32.6	15.42	0.569
Postprandial ISI (10,000/Io x Go)	-8.2	3.35	-21.7	3.87	0.049

IAUC-RI for reflective index. IAUC-SI for stiffness index, IAUC-HR for heart rate, IAUC-MAP for mean arterial pressure, MCP-1: monocyte chemoattractant protein 1, sICAM: soluble intracellular adhesion molecule, data adjusted for age, fat mass, fat free mass, physical activity, A/G ration, seasonal variations and 25(OH)D.

Discussion

Endothelial dysfunction (ED) is a term reflecting the inability of the endothelium to perform its cardinal functions of vasodilation, anti-platelet aggregation and fibrinolysis (264). Therefore, this nexus between vitamin D and endothelial physiology may play a fundamental role in the pathogenesis of cardiovascular disease, particularly in conjunction with MetS. Also, ethnicity is a confounding factor in regards to endothelial function (EF), as it may be impaired in people with darker skin compared to people with lighter skin. A 2004 study compared EF in black African Americans to that in white Americans (265). This study found that in black people, there is upregulated NAD(P)H-oxidase activity which increases oxygen (O2) production. Consequently, these higher levels of O2 reacts with the NO and produce an excess amount of ONOO (265). This causes a greater degree of eNOS uncoupling in black people, whereby the NO bioavailability is made lower (265). As a result, the endothelial cells maintain a homeostasis of NO/O2/ONOO with a similar balance to that of the redox state found in endothelium-impaired function disorders, where there is an excess of ONOO (265). The NO production rate was found to be five times faster in white people, which indicates better EF in white people in comparison to black people (265). Furthermore, a 2005 study comparing endothelial nitric oxide synthase (eNOS) in white American people to eNOS in Brazilian American people found similar ethnic differences (266). That study concluded that the eNOS genetic variance between black and white people does not vary with geographic origin when compared with the previously mentioned study (266).

The aim of this study was to determine the relationship between ethnicity and endothelial function, as measured by PCA, among two ethnic groups: Australian men and Middle Eastern men. This analysis controlled for age, FM, FFM, 25(OH)D, physical activity levels, A/G ratio and seasonal variations. We hypothesised that the those with <50 nmol/L 25(OH)D would demonstrate signs of endothelial dysfunction, indicated by a lesser decrease in RI values compared to the group with sufficient 25(OH)D levels. Accordingly, Middle Eastern men would be expected to have the greater extent of endothelial dysfunction, as they have the lowest 25(OH)D.

In the ANOVA analysis of fasting data, we compared 17 Middle Eastern men to 21 Australian men of European descent, of whom only the Middle Eastern group demonstrated a mean deficiency in 25(OH)D (Table 8). Also, Middle Eastern men were 22 years younger than the Australian men, on average. There were no statistically differences between the groups among basal values of RI, HR, SI, MAP, MCP-1, sICAM and fasting ISI. Fasting SI was higher in Australian men at 8.2 compared to 6.9 in Middle Eastern men, which was against the expected value as the Australian men had higher 25(OH)D values. However, this could be because hyperglycaemia in the fasting state decreases the production of NO through suppression of eNOS activity (267). This is relevant because eNOS activity is the component that varies with ethnicity due to genetic differences. Therefore, ethnic variation did not affect the baseline results which would help us to find the differences between the two groups after the OGTT. Meanwhile, fasting MCP-1 was slightly higher in Australian men, while in contrast, sICAM was slightly higher in Middle Eastern men. Both molecules are reflective of vascular inflammatory status that could lead to endothelial dysfunction. Interestingly, ISI which is reflective of insulin sensitivity (IS) was higher in the Middle Eastern men compared to the Australian men, suggesting some divergence between IS and inflammation in these two groups.

The major focus of the study was the dynamic change in endothelial function in response to glucose ingestion, as in the fasting state, there were no statistically significant markers of EF between the ethnic groups after adjustment for age, FM, FFM, A/G ratio, physical activity and seasonal variations (Table 9). Also, there were no statistically significant changes after adjustment for 25(OH)D (Table 10). We utilized a simple dietary stimulus for two reasons. An OGTT is the standard protocol of assessing glucose intolerance, and hyperglycaemia can produce a reaction that indicates endothelial function and initiate a systemic inflammatory response (268, 269). The MANCOVA analysis showed a decreased level of the adjusted change in the mean of IAUC-RI in the European Australian men compared to Middle Eastern men, two hours after glucose ingestion (Table 9). This lower IAUC-RI, which indicates greater endothelial vasodilation, was consistent with their sufficient 25(OH)D status (211, 212, 233). After adjustment for 25(OH)D and other variables (Table 10), this change in RI

should have been the same between groups if 25OHD accounted for all of EF. Instead some vasodilatory effect in Australian men was still apparent in comparison to Middle Eastern men. This could be related to the better postprandial insulin sensitivity of the Australian men.

There is a general consensus that ongoing hyperglycaemia and diabetes can cause impaired endothelial NO synthesis and action, decreasing the capacity for vascular smooth muscle relaxation (264). Notably, the Australian men demonstrated a high A/G ratio which is a risk factor for CVD (270). The increased A/G ratio was expected to represent a less effective state for glucose homeostasis and therefore decreased ISI responsivity to the OGTT, due to higher glucose and insulin in the blood (264).

Interestingly, there was a decrease in the adjusted change of the mean of IAUC-ISI in the Middle Eastern men compared to the Australian men (Table 10). This result is indicative of a greater postprandial insulin resistance and was a novel observation of our study. However overall it was consistent with the hypothesis that IR would lead to greater endothelial dysfunction and that ethnic groups low in vitamin D status would be most affected. That similar outcome were obtained before and after additional adjustment for 25OHD may suggest an inherent issue of Middle Eastern men in that they have greater postprandial insulin resistance with a trend for this to be manifest as an endothelial dysfunction measured as change in RI.

The change in the adjusted mean of IAUC-HR was higher in Middle Eastern men compared to Australian men, which indicated a greater response to prevent hypotension by increasing cardiac output (stroke volume x heart rate) (271). Also, Middle Eastern men demonstrated a greater decline in sICAM, an endothelial cell activation molecule used with other molecules as indices of as endothelial function. sICAM is expected to have an inverse relationship with endothelial function (26). In this trial we observed worse RI and better sICAM outcomes in Middle Eastern men compared to Australians (Table 8). However another endothelial cell activation molecule, MCP-1 was in the direction of our hypothesis. Clearly we needed a battery of such measures to come to a definite conclusion on endothelial cell activation molecule in response to glucose in Middle Eastern men.

The role of vitamin D in endothelial function is controversial. In our systematic review (chapter 2), we found that vitamin D supplementation might not influence either inflammation or endothelial function. Some literature shows that in the presence of total vitamin D deficiency, vitamin D supplementation improves endothelial function (171). Tarcin et al. (2011) assessed endothelial

physiology using flow mediated dilation (FMD) in asymptomatic patients comparable to our cohort, albeit in a smaller sample size (*n*=23). That study found that total vitamin D deficiency (25(OH)D <50 nmol/L) was associated with less efficient endothelial function. The outcomes of these studies corresponded with most of the evidence in the literature (21, 171, 173-175, 177, 183, 228-232). The present study yielded similar results and controlled for age, 25(OH)D, FFM, FM, seasonal variations and physical activity.

There are related issues that need to be taken into account here. Simply measuring levels of circulating 25(OH)D may not be a sufficient indicator of active vitamin D levels, when differences in the vitamin D binding protein (Vitamin DBP) phenotype among those of different skin colours are taken into consideration (1). Ethnic variations in Vitamin DBP may explain some of the difference between baseline 25(OH)D values in Australian men and Middle Eastern men. As vitamin D is a lipophilic molecule, 99% of it is transported through plasma via Vitamin DBP (1, 272). Nonetheless, the consensus is that bioavailable 25(OH)D is unbound, despite this component of 25(OH)D being extremely minimal (273, 274). It has been demonstrated that Vitamin DBP has a higher affinity for 25(OH)D among some Gc phenotypes, such as in individuals with darker skin pigmentation, which may account for findings of lower unbound plasma 25(OH)D in the Middle Eastern men using conventional assay techniques (1, 275, 276). In fact, Vitamin DBP phenotypes have been shown to account for some differences in circulating 25(OH)D values (1), which are frequently interpreted as vitamin D deficiency in populations with darker pigmented skin (277-279). The phenotypic Vitamin DBP in darker skinned cohorts is often of the higher affinity structure, which suggests that low 25(OH)D results on conventional assays are not reliably representative of deficiency (1). If this is the case, it may explain why the Middle Eastern men demonstrated a slightly better endothelial response to OGTT despite showing lower baseline values of 25(OH)D, as it may not have appropriately captured the bioavailable 25(OH)D concentration in these participants (1). The differences in Gc phenotype among those of different skin colours were not controlled for in our study nor in most of the studies in this field that have examined this relationship.

Also, it has been suggested that 25(OH)D sufficiency should be deemed as values above 75 nmol/L as opposed to the 50 nmol/L threshold, as values below 75 nmol/L may manifest in vitamin D deficiency symptoms (5). This is because the 50 nmol/L threshold was set for bone health and not necessarily for the extra-skeletal functions of vitamin D (5). A recent international consensus paper suggested that 25(OH)D levels between 50-62.5 nmol/L are required for optimal musculoskeletal health (280). This study supports these recommended levels for endothelial function as well, as the Australian men had an adjusted mean of 60.9 nmol/L for 25(OH)D and they displayed satisfactory

endothelial function. However, it is suggested that the target serum 25(OH)D levels should be reviewed to also consider the prevention of other diseases (5).

Study limitations

This study had several limitations. Firstly, in this cross-sectional study the sample size of the Middle Eastern group was small. The lower ages of the Middle Eastern group, although controlled for during analysis, could have influenced the endothelial function outcomes. It is known that endothelial function decreases with age, particularly in men (281-283).

In this study, we used the immunoassay (DiaSorin Liaison) technique which is a popular method for measuring 25(OH)D in the blood. The gold standard for measuring serum 25(OH)D is now LC-MS/MS. However, 25(OH)D measurements by LC-MS/MS vary even among laboratories which use the same technique on the same sample; this may be because of measurement bias and differences in calibration and sample preparation (261).

Finally, we did not collect participant glucose concentrations at the one-hour checkpoint, but only at the baseline and two-hour checkpoints. This information would have been invaluable in determining the effect of OGTT on RI more effectively. The lack of data beyond two hours limited our ability to assess later endothelial function in this study. Therefore, we recommend that future experiments should also assess glucose levels at one hour, assess endothelial function beyond two hours and measure responses to more complex meals. Clearer outcomes could be obtained by measuring more endothelial cell activation molecules like soluble vascular cellular adhesion molecules (sVCAM), P-selectin, E-selectin, hepatocyte growth factor (HGF), nitric oxide (NO) and endothelin-1.

Conclusions

In this cross-sectional study, we examined the endothelial function of two groups, Australian men of European descent and Middle Eastern men, in correlation with 25(OH)D status. We found that vitamin D sufficiency is associated with proficient endothelial function, as measured by PCA (RI). The Middle Eastern men had baseline 25(OH)D deficiency while the Australian men had sufficient 25(OH)D levels by current guidelines. The Australian men demonstrated the better postprandial insulin sensitivity and the more effective endothelial responsivity to OGTT, as they had a better 25(OH)D level. However, the Middle Eastern men displayed slightly better endothelial function than would expected for their low 25(OH)D status. This may be due to ethnic differences in Gc phenotypes (vitamin DBP) which may have resulted in presenting a false lower vitamin D status in the darker skinned Middle Eastern participants. Further research in larger populations is required to

determine if the relationship observed between vitamin D and endothelial function was the effect of 25(OH)D status by itself or also mediated by confounding factors such as ethnicity, age or insulin sensitivity.

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Chapter 6:

Summary & Future Directions

Chapter 6: Summary & Future Directions

Summary of Thesis

The prevalence of Type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) in Australia is rising, and previous studies have indicated an association between low circulating 25(OH)D status and these diseases. Endothelial dysfunction (ED) is a pre-condition that could lead to T2DM and CVD, and thus we suspected that the development of ED may involve vitamin D deficiency. The context of this PhD research was the lack of definitive evidence for a correlation between vitamin D deficiency and endothelial dysfunction (ED). The current cut-off for vitamin D sufficiency is circulating 25(OH)D $\geq$ 50 nmol/L, which was set with regards to bone health (5). However, in this thesis we questioned whether a higher cut-off for sufficiency could prevent endothelial dysfunction.

There is a relationship between the systemic inflammation and vascular pathology that lead to endothelial dysfunction (ED). This, in turn, may underscore insulin resistance (IR), and its subsequent progression to T2DM and CVD. These diseases are generally associated with obesity (19). Higher fat mass allows increased storage of fat-soluble vitamins such as 25(OH)D, thereby resulting in a lower 25(OH)D status (20). 25(OH)D plays an important role in the regulation of endothelial function (EF) (11, 35, 44-51), and EF subsequently affects the movement of insulin across the capillaries, which means that ED may result in IR (29). Also, impairment of the function of the vitamin D receptors (VDRs) on pancreatic cells may lower insulin production, which would then alter the balance between vasodilators and vasoconstrictors, thus causing ED (29).

In this thesis, I firstly examined the relationship between 25(OH)D and endothelial function in a literature review. I also conducted two cross-sectional studies: the first study to evaluate the effect of 25(OH)D status on EF and lipid profiles among Australian adults, and the second study to compare Australian of European descent adults to Middle Eastern adults in terms of 25(OH)D status and its effects on ED. I found a consistent effect of vitamin D status on endothelial function or markers of dyslipidaemia and endothelial cell activation. This prompted me to more thoroughly investigate whether a causative role of vitamin D was detectable. I did so through a systematic review of RCTs, with the aim of finding the optimal level of 25(OH)D that I could propose would be necessary to avert ED and thus T2DM and CVD. Unfortunately, we did not have sufficient time and money to properly examine this relationship in a RCT of our own.

In the literature review and the review paper, we found many theories linking 25(OH)D to ED through many physiological pathways by means of VDRs spread throughout the human body. These

VDRs are involved in the regulation of blood pressure, the prevention of arterial calcification and the suppression of inflammatory states (11, 35, 44-51). Recent research has suggested that 25(OH)D ≥ 75 nmol/L is required for optimal bone health, which indicates that the requirement for the extra-skeletal benefits of 25(OH)D may be even higher (5). There is now a suggestion that the IOM report on RDA for vitamin D may have been incorrect and this adds an uncertainty factor to global RDAs (284). If confirmed, this would mean everyone needs more vitamin D which would elevate the normal circulating level even higher. A few trials supported the role of vitamin D supplementation in improving EF. Meanwhile, most of the trials did not show vitamin D supplementation to have an effect on EF. However, the initial 25(OH)D levels, the doses of vitamin D and insufficient maintenance periods of the optimum level of 25(OH)D might have resulted in false negative results regarding the 25(OH)D levels that are beneficial for EF (26).

The first cross-sectional study examined the relationship of 25(OH)D to dyslipidaemia and endothelial cell activation molecules. One hundred and one Australian adults were recruited for this study with the result that 83 participants with completed data were analysed. Then they were divided into three tertiles according to their 25(OH)D levels, where the lower group had a mean of 56.8 nmol/L, the moderate group had a mean of 74.5 nmol/L and the higher group had a mean of 97 nmol/L. As a result, we found a beneficial effect of the higher level of 25(OH)D on total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) compared to the lower 25(OH)D level. Also, we found a beneficial effect on the moderate level of 25(OH)D on triglycerides (TG) compared to the lower 25(OH)D level. Furthermore, we found a deleterious effect of the higher level of 25(OH)D on soluble vascular cell adhesion molecules (sVCAM) compared to the lower 25(OH)D group, as increased levels of sVCAM are an early sign of inflammation. Finally, there were high levels of hepatocyte growth factor (HGF) in the higher 25(OH)D group compared to the lower group. These may have increased to counteract the higher levels of inflammation, as indicated by the increased levels of sVCAM.

The second cross-sectional study examined the relationship between 25(OH)D status and EF among 38 participants from two ethnic groups: 17 Middle Eastern men and 21 Australians. Levels of IR were measured by the 75-g OGTT and the Sluiter et al equation for ISI (10000/(Insulin*glucose)) in the fasting state. EF was measured by the Pulse Contour Analysis (PCA) method using the Digital Volume Pulse Trace (PT-2000) device. This was conducted after the participants were all given the same type of low-sodium, low-fat meal on the night prior to the test, fasting on the morning of the test and then being administered glucose. We found the 25(OH)D levels were 60.9 nmol/L in the Australian men of European descent and 40.4 nmol/L in the Middle Eastern men. The Australian

men displayed the better endothelial function test results. This result was expected as the Australian men had sufficient 25(OH)D levels and better insulin sensitivity compared with the Middle Eastern men. While 25(OH)D made an independent contribution to endothelial function, this study showed a difference among the ethnic groups even after adjustment for several confounders. One possible contribution may be the group specific-globulin (Gc) phenotypes of the 25(OH)D carrier (the vitamin D binding protein (Vitamin DBP)) which are predominantly found in individuals with darker skin (1).

The systematic review we conducted found 14 studies which fulfilled our criteria for establishing the relationship between cholecalciferol supplementation and EF, endothelial cells activation molecules and inflammatory biomarkers. Therein we found only three trials that supported the role of cholecalciferol in improving EF, one study that supported the role of cholecalciferol supplementation in improving the levels of C-reactive protein (CRP), and no studies that supported the role of cholecalciferol supplementation on improving endothelial cell activation molecules. We found no consistent optimum level of 25(OH)D in those studies supporting a role in EF, however an improvement in EF was found when 25(OH)D levels in two of the trials were increased to approximately 50 nmol/L, which is the current cut-off value, and the 25(OH)D level in the other trial was increased to above 100 nmol/L. In the trial that showed an improvement in CRP, the 25(OH)D increased from 49 to 62 nmol/L. These findings did not support the recent suggestion that levels of 25(OH)D $\geq$ 75 nmol/L may be required. Instead, they suggest that the current cut-off 50 nmol/L may be sufficient to improve endothelial function (5).

Implications for future research

These findings have led us to develop a systematic study protocol in this area which could be used as a guideline for future research into the effects of vitamin D supplementation on EF. This includes the measurement of EF directly through endothelial cell activation molecules as well as indirectly through inflammatory biomarkers.

Recent data showed that 23% of the Australian adult population experience vitamin D deficiency (circulating 25(OH)D <50 nmol/L) with women and the elderly being more at risk than the general population (4, 5, 99, 100). Therefore, this population may be at a greater risk of developing endothelial dysfunction. There is an urgent need for international and Australian expert committees to revisit the adequate intake (AI) for vitamin D intake as it is significantly below the current US RDA for vitamin D intake. For example, for adults aged 19-50 years old, the Australian AI is 5 μg compared to the RDA which is 15 μg.

Complex interrelationships between vitamin D, endothelial function (EF), IR, obesity, metabolic syndrome and the progression of T2DM and CVD are indicated in the literature, yet the exact interactions between these processes and states are unclear. Australia is a multi-cultural country and many ethnic groups have increased susceptibility to IR/T2DM even though they are certified healthy when they migrate to the country. Therefore, a RCT of a large sample of people including at least two ethnic groups for an extended period of time is needed to determine the exact nature of the relationship between 25(OH)D status and EF.

Further research should aim to find the optimum level of 25(OH)D for extra-skeletal benefit. One way forward could be through a long term high quality RCT, with an active control. For example, people with mild vitamin D deficiency (25(OH)D 30-50 nmol/L), could be randomised to receive 1000, 2000, 3000, 4000 IU/day over a year such that they maintain their 25(OH)D levels within one of four ranges 25(OH)D within 30-50 nmol/L, second group maintains 25(OH)D within 50-65 nmol/L, third group maintains 25(OH)D within 65-80 nmol/L and the final group maintains 25(OH)D >80 nmol/L. Once the 25(OH)D levels of the participants within each group are all within the appropriate range, they should be consistently maintained within that ranges for the subsequent 12 months. During this time, the following outcomes should be measured on a 3 monthly basis: endothelial function (EF), insulin resistance (IR), insulin sensitivity index (ISI), lipid profiles, systemic inflammatory biomarkers, endothelial cell activations molecules and parathyroid hormone (PTH).

Future studies should evaluate EF using a combination of techniques with high validity and reliability, which would all be non-invasive to make them more tolerable for the participants. An example of such an evaluation method is the use of flow-mediated dilatation (FMD) as well as pulse contour analysis (PCA) and pulse wave velocity (PWV) to help to consolidate the outcomes/results. Also, clinical assessment of dynamic changes in endothelial function could use an agent such as acetylcholine or salbutamol to produce more accurate measurements (56).

Body weight should be consistently monitored and maintained within reasonable limits throughout the study so as to avoid 25(OH)D sequestering into the fat cells in obese participants (236). Also, the levels of physical activity and sun exposure in the placebo group should be controlled for so that the amount of vitamin D intake can be maintained throughout the study. The analysis of the results would need to consider the possibility of the difference in the Gc phenotype of the vitamin D binding protein (Vitamin DBP) in the participants of darker skin pigmentation. There is evidence that the

Vitamin DBP phenotypes in people with darker skin may have higher affinity for binding to 25(OH)D, therapy reducing the amount of circulating 25(OH)D (285). However, the 25(OH)D that is bound to the Vitamin DBP is reabsorbed into endocytic receptors where it is converted to the active form $1,25(OH)_2D$ (286). This absorption may also vary in efficacy according to Vitamin DBP phenotype.

References

1. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, et al. Vitamin D–binding protein and vitamin D status of black Americans and white Americans. N Engl J Med. 2013;369(21):1991-2000.
2. Mithal A, Wahl D, Bonjour J-P, Burckhardt P, Dawson-Hughes B, Eisman J, et al. Global vitamin D status and determinants of hypovitaminosis D. Osteoporos Int. 2009;20(11):1807-20.
3. Australian Bureau of Statistics Australian Health Survey: Users' Guide, 2011-13 Australia: ABS,; 2013.
4. Australian Health Survey: Biomedical Results for Nutrients, 2011-12 [Internet]. Australian Bureau of Statistics 2012 [cited 19-04-2016]. Available from: http://www.abs.gov.au/ausstats/abs@.nsf/Lookup/4364.0.55.006Chapter2002011-12.
5. Nowson CA, McGrath JJ, Ebeling PR, Haikerwal A, Daly RM, Sanders KM, et al. Vitamin D and health in adults in Australia and New Zealand: a position statement. Med J Aust. 2012;196(11):686-7.
6. Danescu LG, Levy S, Levy J. Vitamin D and diabetes mellitus. Endocrine. 2009;35(1):11-7.
7. Pasco JA, Henry MJ, Nicholson GC, Sanders KM, Kotowicz MA. Vitamin D status of women in the Geelong Osteoporosis Study: association with diet and casual exposure to sunlight. Med J Aust. 2001;175(8):401-6.
8. World Health Organisation WHO. Global status report on noncommunicable diseases. 2010.
9. Gordon K, Smith F, Dhillon S. Effective chronic disease management: patients' perspectives on medication-related problems. Patient Educ Couns. 2007;65(3):407-15.
10. Statistics ABo. National Health Survey: First Results, 2014-15 Canberra: Australian Bureau of Statistics; 2015 [updated 08/12/2015 Available from: http://www.abs.gov.au/Health-Conditions-and-Risk-Factors.
11. Sorisky A. Molecular links between obesity and cardiovascular disease. Am J Ther. 2002;9(6):516-21.
12. Scheme NDS. Diabetes Australia 2016 [updated 30/09/2016. Available from: http://www.ndss.com.au/diabetes-map/.
13. Lindström J, Peltonen M, Eriksson J, Ilanne-Parikka P, Aunola S, Keinänen-Kiukaanniemi S, et al. Improved lifestyle and decreased diabetes risk over 13 years: long-term follow-up of the randomised Finnish Diabetes Prevention Study (DPS). Diabetologia. 2013;56(2):284-93.
14. Schwarz P, Lindström J, Kissimova-Scarbeck K, Szybinski Z, Barengo N, Peltonen M, et al. The European perspective of type 2 diabetes prevention: diabetes in Europe-prevention using lifestyle, physical activity and nutritional intervention (DE-PLAN) project. Exp Clin Endocrinol Diabetes. 2008;116(03):167-72.
15. Gloyn AL, Braun M, Rorsman P. Type 2 diabetes susceptibility gene TCF7L2 and its role in β-Cell function. Diabetes. 2009;58(4):800-2.
16. Dou H, Ma E, Yin L, Jin Y, Wang H. The association between gene polymorphism of TCF7L2 and type 2 diabetes in Chinese Han population: a meta-analysis. PloS one. 2013;8(3):e59495.
17. Reiling E, van Vliet-Ostaptchouk JV, Van't Riet E, Van Haeften TW, Arp PA, Hansen T, et al. Genetic association analysis of 13 nuclear-encoded mitochondrial candidate genes with type II diabetes mellitus: the DAMAGE study. Eur J Hum Genet. 2009;17(8):1056-62.
18. Reiling E, Van't Riet E, Groenewoud M, Welschen L, Van Hove E, Nijpels G, et al. Combined effects of single-nucleotide polymorphisms in GCK, GCKR, G6PC2 and MTNR1B on fasting plasma glucose and type 2 diabetes risk. Diabetologia. 2009;52(9):1866-70.
19. Castro DC, Samuels M, Harman AE. Growing Healthy Kids: A Community Garden–Based Obesity Prevention Program. Am J Prev Med 2013;44(3):S193-S9.
20. Foss Y. Vitamin D deficiency is the cause of common obesity. Med Hypotheses. 2009;72(3):314-21.
21. Tarcin O, Yavuz DG, Ozben B, Telli A, Ogunc AV, Yuksel M, et al. Effect of vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. J Clin Endocrinol Metab. 2009;94(10):4023-30.
22. Borissova A, Tankova T, Koev D. Insulin secretion, peripheral insulin sensitivity and insulin-receptor binding in subjects with different degrees of obesity. Diabetes Metab. 2004;30(5):425-31.

23. Zeitz U, Weber K, Soegiarto DW, Wolf E, Balling R, Erben RG. Impaired insulin secretory capacity in mice lacking a functional vitamin D receptor. FASEB J. 2003;17(3):509-11.
24. Soares M, Ping-Delfos WCS, Ghanbari M. Calcium and vitamin D for obesity: a review of randomized controlled trials. Euro J Clin Nutr. 2011;65(9):994-1004.
25. Soares M, Patakh K. Vitamin D supplementation for obesity: potential mechanisms of action and an update of randomized controlled trials. Curr Nutr Food Sci 2014;10(1):29-35.
26. Alyami A, Soares M, Sherriff J, Mamo J. Vitamin D & endothelial function. Indian J Med Res. 2014;140(4):483.
27. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet. 2005;365(9468):1415–28.
28. Wang CC, Goalstone ML, Draznin B. Molecular mechanisms of insulin resistance that impact cardiovascular biology. Diabetes. 2004;53(11):2735–40.
29. Cersosimo E, DeFronzo RA. Insulin resistance and endothelial dysfunction: the road map to cardiovascular diseases. Diabetes Metab Res Rev. 2006;22(6):423-36.
30. Lu H, Daugherty A. Recent Highlights of ATVB Atherosclerosis. Arterioscler Thromb Vasc Biol. 2015;35(3):485.
31. Sitia S, Tomasoni L, Atzeni F, Ambrosio G, Cordiano C, Catapano A, et al. From endothelial dysfunction to atherosclerosis. Autoimmun Rev. 2010;9(12):830-4.
32. Yang Z, Ming X-F. Recent advances in understanding endothelial dysfunction in atherosclerosis. Clin Med Res. 2006;4(1):53-65.
33. Haussler MR, Haussler CA, Bartik L, Whitfield GK, Hsieh J-C, Slater S, et al. Vitamin D receptor: molecular signaling and actions of nutritional ligands in disease prevention. Nutr Rev. 2008;66(suppl 2):S98-S112.
34. Zehnder D, Bland R, Chana RS, Wheeler DC, Howie AJ, Williams MC, et al. Synthesis of 1, 25-dihydroxyvitamin D3 by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. J Am Soc Nephrol. 2002;13(3):621-9.
35. Norman P, Powell J. Vitamin D, shedding light on the development of disease in peripheral arteries. Arterioscler Thromb Vasc Biol. 2005;25(1):39-46.
36. Sasso FC, Torella D, Carbonara O, Ellison GM, Torella M, Scardone M, et al. Increased vascular endothelial growth factor expression but impaired vascular endothelial growth factor receptor signaling in the myocardium of type 2 diabetic patients with chronic coronary heart disease. J Am Coll Cardiol. 2005;46(5):827-34.
37. Ford ES, Zhao G, Li C, Pearson WS. Serum concentrations of vitamin D and parathyroid hormone and prevalent metabolic syndrome among adults in the United States. J Diabetes. 2009;1(4):296-303.
38. Kim MK, Il Kang M, Won Oh K, Kwon HS, Lee JH, Lee WC, et al. The association of serum vitamin D level with presence of metabolic syndrome and hypertension in middle-aged Korean subjects. Clin Endocrinol (Oxf). 2010;73(3):330-8.
39. Lee DM, Rutter MK, O'Neill TW, Boonen S, Vanderschueren D, Bouillon R, et al. Vitamin D, parathyroid hormone and the metabolic syndrome in middle-aged and older European men. Eur J Endocrinol. 2009;161(6):947-54.
40. Soares M, Ping-Delfos WCS, Sherriff J, Nezhad D, Cummings N, Zhao Y. Vitamin D and parathyroid hormone in insulin resistance of abdominal obesity: cause or effect? Euro J Clin Nutr. 2011;65(12):1348-52.
41. Cigolini M, Iagulli MP, Miconi V, Galiotto M, Lombardi S, Targher G. Serum 25-hydroxyvitamin D3 concentrations and prevalence of cardiovascular disease among type 2 diabetic patients. Diabetes Care. 2006;29(3):722-4.
42. Pannu PK, Calton EK, Soares MJ. Chapter Two-Calcium and Vitamin D in Obesity and Related Chronic Disease. Adv Food Nutr Res. 2016;77:57-100.
43. Guillot X, Semerano L, Saidenberg-Kermanac'h N, Falgarone G, Boissier M-C. Vitamin D and inflammation. Joint Bone Spine. 2010;77(6):552-7.
44. Li YC, Kong J, Wei M, Chen Z-F, Liu SQ, Cao L-P. 1, 25-Dihydroxyvitamin D 3 is a negative endocrine regulator of the renin-angiotensin system. J Clin Invest. 2002;110(2):229-38.

45. Tousoulis D, Kampoli A-M, Tentolouris Nikolaos Papageorgiou C, Stefanadis C. The role of nitric oxide on endothelial function. Curr Vasc Pharmacol. 2012;10(1):4-18.
46. Goodfellow J, Owens D, Henderson A. Cardiovascular Syndromes X, endothelial dysfunction and insulin resistance. Diabetes Res Clin Pract. 1996;31(Supplement 1):S163-S71.
47. Barac A, Campia U, Panza JA. Methods for evaluating endothelial function in humans. Hypertension. 2007;49(4):748-60.
48. Vogel RA. Measurement of endothelial function by brachial artery flow-mediated vasodilation. Am J Cardiol. 2001;88(2):31-4.
49. Watson KE, Abrolat ML, Malone LL, Hoeg JM, Doherty T, Detrano R, et al. Active serum vitamin D levels are inversely correlated with coronary calcification. Circulation. 1997;96(6):1755-60.
50. Schleithoff SS, Zittermann A, Tenderich G, Berthold HK, Stehle P, Koerfer R. Vitamin D supplementation improves cytokine profiles in patients with congestive heart failure: a double-blind, randomized, placebo-controlled trial. Am J Clin Nutr. 2006;83(4):754-9.
51. Sadeghi K, Wessner B, Laggner U, Ploder M, Tamandl D, Friedl J, et al. Vitamin D3 down-regulates monocyte TLR expression and triggers hyporesponsiveness to pathogen-associated molecular patterns. Eur J Immunol. 2006;36(2):361-70.
52. D'Aurizio F, Villalta D, Metus P, Doretto P, Tozzoli R. Is vitamin D a player or not in the pathophysiology of autoimmune thyroid diseases? Autoimmun Rev. 2015;14(5):363-9.
53. Pasimeni G, Ribaudo MC, Capoccia D, Rossi F, Bertone C, Leonetti F, et al. Non-invasive evaluation of endothelial dysfunction in uncomplicated obesity: Relationship with insulin resistance. Microvasc Res. 2006;71(2):115-20.
54. Tousoulis D, Antoniades C, Stefanadis C. Evaluating endothelial function in humans: a guide to invasive and non-invasive techniques. Heart. 2005;91(4):553-8.
55. Woodman R, Watts G. Measurement and application of arterial stiffness in clinical research: focus on new methodologies and diabetes mellitus. Med Sci Monit. 2003;9(5):RA81-RA9.
56. Millasseau SC, Ritter JM, Takazawa K, Chowienczyk PJ. Contour analysis of the photoplethysmographic pulse measured at the finger. J Hypertens. 2006;24(8):1449-56.
57. Rajakumar K. Vitamin D, cod-liver oil, sunlight, and rickets: a historical perspective. Pediatrics. 2003;112(2):e132-e5.
58. Wolf G. The discovery of vitamin D: the contribution of Adolf Windaus. J Nutr. 2004;134(6):1299-302.
59. IOM (Institue of Medicine). Dietary Reference Intakes for Calcium and Vitamin D. In: Press TNA, editor. Washington, DC2011. p. 75-111.
60. Nowson CA, Sambrook P, Diamond T, Pasco J, Mason R, Eisman JA. Vitamin D in Australia: issues and recommendations. Aust Fam Physician. 2004;33(3):133.
61. Joshi D, Center JR, Eisman JA. Vitamin D deficiency in adults. Aust Prescr. 2010;33(4):103-6.
62. Nozza JM, Rodda CP. Vitamin D deficiency in mothers of infants with rickets. Med J Aust. 2001;175(5):253.
63. Borel P, Caillaud D, Cano N. Vitamin D bioavailability: state of the art. Crit Rev Food Sci Nutr. 2015;55(9):1193-205.
64. ABS. Australian Health Survey: Biomedical results for nutrients. In: ABS, editor. Canberra2014.
65. Black L, Jacoby P, Nowson C, Daly R, Lucas R. Predictors of Vitamin D-Containing Supplement Use in the Australian Population and Associations between Dose and Serum 25-Hydroxyvitamin D Concentrations. Nutrients. 2016;8(6).
66. Pasco JA, Henry MJ, Nicholson GC, Sanders KM, Kotowicz MA. Vitamin D status of women in the Geelong Osteoporosis Study: association with diet and casual exposure to sunlight. Med J Aust. 2001;175(8):401-5.
67. Nationnal Health and Medical Research Council. Nutrient Reference Values for Australia and New Zealand. Australia: Ministry fo Health; 2014.
68. Nationnal Institutes of Health. Vitamin D fact sheet for health professionals. 2016.

69. Pludowski P, Holick MF, Pilz S, Wagner CL, Hollis BW, Grant WB, et al. Vitamin D effects on musculoskeletal health, immunity, autoimmunity, cardiovascular disease, cancer, fertility, pregnancy, dementia and mortality—a review of recent evidence. Autoimmun Rev. 2013;12(10):976-89.
70. Zella LA, Shevde NK, Hollis BW, Cooke NE, Pike JW. Vitamin D-binding protein influences total circulating levels of 1, 25-dihydroxyvitamin D3 but does not directly modulate the bioactive levels of the hormone in vivo. Endocrinology. 2008;149(7):3656-67.
71. Cannell JJ, Grant WB, Holick MF. Vitamin D and inflammation. Derma Endocrinol. 2014;6(1):e983401.
72. Prietl B, Treiber G, Pieber TR, Amrein K. Vitamin D and immune function. Nutrients. 2013;5(7):2502-21.
73. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science. 2006;311(5768):1770-3.
74. Zaiou M, Gallo RL. Cathelicidins, essential gene-encoded mammalian antibiotics. J Mol Med (Berl). 2002;80(9):549-61.
75. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. J Leukoc Biol. 2004;75(1):39-48.
76. Wang T-T, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1, 25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. J Immunol. 2004;173(5):2909-12.
77. Adams JS, Ren S, Liu PT, Chun RF, Lagishetty V, Gombart AF, et al. Vitamin D-directed rheostatic regulation of monocyte antibacterial responses. J Immunol. 2009;182(7):4289-95.
78. Weber F. Absorption mechanisms for fat-soluble vitamins and the effect of other food constituents. Prog Clin Biol Res. 1981;77:119-35.
79. Weber F. Absorption of fat-soluble vitamins. Int J Vit Nutr Res. 1980;25:55.
80. Johnson J, Mistry V, Vukovich M, Hogie-Lorenzen T, Hollis B, Specker B. Bioavailability of vitamin D from fortified process cheese and effects on vitamin D status in the elderly. J Dairy Sci. 2005;88(7):2295-301.
81. Mulligan GB, Licata A. Taking vitamin D with the largest meal improves absorption and results in higher serum levels of 25-hydroxyvitamin D. J Bone Miner Res. 2010;25(4):928-30.
82. Thompson G, Lewis B, Booth C. Absorption of vitamin D3-3H in control subjects and patients with intestinal malabsorption. J Clin Invest. 1966;45(1):94.
83. Blomstrand R, Forsgren L. Intestinal absorption and esterification of vitamin D3-1, 2-3H in man. Acta Chem Scand. 1967;21(6):1662-3.
84. Compston JE, Merrett AL, Hammett F, Magill P. Comparison of the appearance of radiolabelled vitamin D3 and 25-hydroxy-vitamin D3 in the chylomicron fraction of plasma after oral administration in man. Clin Sci (Lond). 1981;60(2):241-3.
85. Jones G. Pharmacokinetics of vitamin D toxicity. Am J Clin Nutr 2008;88(2):582S-6S.
86. Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW. De-orphanization of Cytochrome P450 2R1 a microsomal vitamin D 25-hydroxylase. J Biol Chem. 2003;278(39):38084-93.
87. Strushkevich N, Usanov SA, Plotnikov AN, Jones G, Park H-W. Structural analysis of CYP2R1 in complex with vitamin D 3. J Mol Biol. 2008;380(1):95-106.
88. Wang TJ, Zhang F, Richards JB, Kestenbaum B, Van Meurs JB, Berry D, et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. Lancet. 2010;376(9736):180-8.
89. Laing CJ, Cooke NE. Vitamin D-binding protein. Br J Nutr. 2005;88(2):117-34.
90. Tanaka YaHFD. Stimulation of 1,25-dihydroxyvitamin D3 production by 1,25-dihydroxyvitamin D3 in the hypocalcaemic rat. Biochem J. 1983;214(3):893-7.
91. Blum M, Dallal GE, Dawson-Hughes B. Body size and serum 25 hydroxy vitamin D response to oral supplements in healthy older adults. J Am Coll Nutr. 2008;27(2):274-9.
92. LIEL Y, EDWARDS J, SHARY J, SPICER KM, GORDON L, BELL NH. The Effects of Race and Body Habitus on Bone Mineral Density of the Radius, Hip, and Spine in Premenopausal Women*. J Clin Endocrinol Metab. 1988;66(6):1247-50.

93. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. Am J Clin Nutr. 2000;72(3):690-3.
94. Hayes A, Lung T, Bauman A, Howard K. Modelling obesity trends in Australia: unravelling the past and predicting the future. Int J Obes (Lond). 2017;41(1):178-85.
95. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357(3):266-81.
96. Vieth R. Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. Am J Clin Nutr. 1999;69(5):842-56.
97. Schleicher RL, Sternberg MR, Lacher DA, Sempos CT, Looker AC, Durazo-Arvizu RA, et al. The vitamin D status of the US population from 1988 to 2010 using standardized serum concentrations of 25-hydroxyvitamin D shows recent modest increases. Am J Clin Nutr. 2016;104(2):454-61.
98. van der Mei IA, Ponsonby A-L, Engelsen O, Pasco JA, McGrath JJ, Eyles DW, et al. The high prevalence of vitamin D insufficiency across Australian populations is only partly explained by season and latitude. Environ Health Perspect. 2007:1132-9.
99. Sambrook PN, Cameron ID, Cumming RG, Lord SR, Schwarz JM, Trube A, et al. Vitamin D deficiency is common in frail institutionalised older people in northern Sydney. Med J Aust. 2002;176(11):560-.
100. Inderjeeth C, Barrett T, Al-Lahham Y, Mulford J, Nicklason F, Reberger C. Seasonal variation, hip fracture and vitamin D levels in Southern Tasmania. N Z Med J. 2002;115(1152):183-5.
101. Inderjeeth C, Nicklason F, Al-Lahham Y, Greenaway T, Jones G, Parameswaran V, et al. Vitamin D deficiency and secondary hyperparathyroidism: clinical and biochemical associations in older non-institutionalised Southern Tasmanians. Aust N Z J Med. 2000;30(2):209-14.
102. McAuley K, Jones S, Lewis-Barned N, Manning P, Goulding A. Low vitamin D status is common among elderly Dunedin women. N Z Med J. 1997;110(1048):275-7.
103. Bolland MJ, Grey AB, Ames RW, Mason BH, Horne AM, Gamble GD, et al. The effects of seasonal variation of 25-hydroxyvitamin D and fat mass on a diagnosis of vitamin D sufficiency. Am J Clin Nutr. 2007;86(4):959-64.
104. Green TJ, Skeaff CM, Rockell JE, Venn BJ, Lambert A, Todd J, et al. Vitamin D status and its association with parathyroid hormone concentrations in women of child-bearing age living in Jakarta and Kuala Lumpur. Eur J Clin Nutr. 2008;62(3):373-8.
105. Arya V, Bhambri R, Godbole MM, Mithal A. Vitamin D status and its relationship with bone mineral density in healthy Asian Indians. Osteoporos Int. 2004;15(1):56-61.
106. Zargar A, Ahmad S, Masoodi S, Wani A, Bashir M, Laway B, et al. Vitamin D status in apparently healthy adults in Kashmir Valley of Indian subcontinent. Postgrad Med J. 2007;83(985):713-6.
107. Sachan A, Gupta R, Das V, Agarwal A, Awasthi PK, Bhatia V. High prevalence of vitamin D deficiency among pregnant women and their newborns in northern India. Am J Clin Nutr. 2005;81(5):1060-4.
108. Puri S, Marwaha RK, Agarwal N, Tandon N, Agarwal R, Grewal K, et al. Vitamin D status of apparently healthy schoolgirls from two different socioeconomic strata in Delhi: relation to nutrition and lifestyle. Br J Nutr. 2008;99(04):876-82.
109. Goswami R, Gupta N, Goswami D, Marwaha RK, Tandon N, Kochupillai N. Prevalence and significance of low 25-hydroxyvitamin D concentrations in healthy subjects in Delhi. Am J Clin Nutr. 2000;72(2):472-5.
110. Atiq M, Suria A, Nizami S, Ahmed I. Vitamin D status of breastfed Pakistani infants. Acta Pædiatr. 1998;87(7):737-40.
111. Rashid A, Mohammed T, Stephens W, Warrington S, Berry J, Mawer E. Vitamin D state of Asians living in Pakistan. Br Med J (Clin Res Ed). 1983;286(6360):182-4.
112. Islam M, Lamberg-Allardt C, Karkkainen M, Outila T, Salamatullah Q, Shamim A. Original Communications-Vitamin D deficiency: A concern in premenopausal Bangladeshi women of two socio-economic groups in rural and urban region. Euro J Clin Nutr. 2002;56(1):51-6.
113. Rodrigo M. Peak bone mass measured by phalangeal BMD and its association with nutritional status, socioeconomics status and physical activity: a community based cross sectional study in Galle district, Sri Lanka. Extract from PHD thesis (unpublished). 2007.

114. Fraser DR. Vitamin D-deficiency in Asia. J Steroid Biochem Mol Biol. 2004;89(90):491-5.
115. Foo L, Zhang Q, Zhu K, Greenfield H, Fraser D. Vitamin D status and its relationship with bone mass in healthy adolescent girls in China. Asia Pac J Clin Nutr. 2005;14:S40-S.
116. Yan L, Prentice A, Zhang H, Wang X, Stirling D, Golden M. Vitamin D status and parathyroid hormone concentrations in Chinese women and men from north-east of the People's Republic of China. Eur J Clin Nutr. 2000;54(1):68-72.
117. Nakamura K, Nashimoto M, Matsuyama S, Yamamoto M. Low serum concentrations of 25-hydroxyvitamin D in young adult Japanese women: a cross sectional study. Nutrition. 2001;17(11):921-5.
118. Arneil G. Nutritional rickets in children in Glasgow. Proc Nutr Soc. 1975;34(2):101-9.
119. Holmes A, Enoch B, Taylor J, Jones M. Occult rickets and osteomalacia amongst the Asian immigrant population. Q J Med. 1972;42(165):125-49.
120. Oliveri B, Plantalech L, Bagur A, Wittich A, Rovai G, Pusiol E, et al. High prevalence of vitamin D insufficiency in healthy elderly people living at home in Argentina. Eur J Clin Nutr. 2004;58(2):337-42.
121. Lips P, Hosking D, Lippuner K, Norquist J, Wehren L, Maalouf G, et al. The prevalence of vitamin D inadequacy amongst women with osteoporosis: an international epidemiological investigation. J Intern Med. 2006;260(3):245-54.
122. Looker AC, Johnson, C. L., Lacher, D. A., Pfeiffer, C. M., Schleicher, R. L., & Sempos, C. T. Vitamin D status: United states, 2001–2006. NCHS data brief. 2011;59(1):1-8.
123. Vieth R, Cole D, Hawker G, Trang H, Rubin L. Original Communications-Wintertime vitamin D insufficiency is common in young Canadian women, and their vitamin D intake does not prevent it. Euro J Clin Nutr. 2001;55(12):1091-7.
124. Liu BA, Gordon M, Labranche JM, Murray TM, Vieth R, Shear NH. Seasonal prevalence of vitamin D deficiency in institutionalized older adults. J Am Geriatr Soc. 1997;45(5):598-603.
125. Cashman KD, Dowling KG, Škrabáková Z, Gonzalez-Gross M, Valtueña J, De Henauw S, et al. Vitamin D deficiency in Europe: pandemic? Am J Clin Nutr. 2016;103(4):1033-44.
126. Moreno L, De Henauw S, Gonzalez-Gross M, Kersting M, Molnar D, Gottrand F, et al. Design and implementation of the healthy lifestyle in Europe by nutrition in adolescence cross-sectional study. Int J Obes (Lond). 2008;32(Suppl 5):S4-S11.
127. González-Gross M, Valtuena J, Breidenassel C, Moreno LA, Ferrari M, Kersting M, et al. Vitamin D status among adolescents in Europe: the Healthy Lifestyle in Europe by Nutrition in Adolescence study. Br J Nutr. 2012;107(05):755-64.
128. Kurth B-M, Kamtsiuris P, Hölling H, Schlaud M, Dölle R, Ellert U, et al. The challenge of comprehensively mapping children's health in a nation-wide health survey: design of the German KiGGS-Study. BMC Public health. 2008;8(1):1.
129. Scheidt-Nave C, Kamtsiuris P, Gößwald A, Hölling H, Lange M, Busch MA, et al. German health interview and examination survey for adults (DEGS)-design, objectives and implementation of the first data collection wave. BMC Public health. 2012;12(1):1.
130. Kamtsiuris P, Lange M, Hoffmann R, Rosario AS, Dahm S, Kuhnert R, et al. The first wave of the German Health Interview and Examination Survey for Adults (DEGS1). Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz. 2013s;56(5/6):620-30.
131. National Diet and Nutrition Survey: Headline results from Years 1 to 4 (combined) of the rolling programme from 2008 and 2009 to 2011 and 2012. 2014 [Internet]. [cited 09/05/2016]. Available from: https://www.gov.uk/government/publications/national-diet-and-nutrition-survey-results-from-years-1-to-4-combined-of-the-rolling-programme-for-2008-and-2009-to-2011-and-2012.
132. O'Donovan SM, Murray DM, Hourihane JOB, Kenny LC, Irvine AD, Kiely M. Cohort profile: the Cork BASELINE Birth Cohort Study: babies after SCOPE: evaluating the longitudinal impact on neurological and nutritional endpoints. Int J Epidemiol. 2015;44(3):764-75.
133. Cashman KD, Kiely M, Kinsella M, Durazo-Arvizu RA, Tian L, Zhang Y, et al. Evaluation of Vitamin D Standardization Program protocols for standardizing serum 25-hydroxyvitamin D data: a

case study of the program's potential for national nutrition and health surveys. Am Clin Nutr 2013;97(6):1235-42.
134. Harris TB, Launer LJ, Eiriksdottir G, Kjartansson O, Jonsson PV, Sigurdsson G, et al. Age, Gene/Environment Susceptibility–Reykjavik Study: multidisciplinary applied phenomics. Am J Epidemiol. 2007;165(9):1076-87.
135. Damsgaard CT, Dalskov S-M, Petersen RA, Sørensen LB, Mølgaard C, Biltoft-Jensen A, et al. Design of the OPUS School Meal Study: a randomised controlled trial assessing the impact of serving school meals based on the New Nordic Diet. Scand J Public Health. 2012;40(8):693-703.
136. Damsgaard CT, Dalskov S-M, Laursen RP, Ritz C, Hjorth MF, Lauritzen L, et al. Provision of healthy school meals does not affect the metabolic syndrome score in 8–11-year-old children, but reduces cardiometabolic risk markers despite increasing waist circumference. Br J Nutr. 2014;112(11):1826-36.
137. Cashman KD, Dowling KG, Škrabáková Z, Kiely M, Lamberg-Allardt C, Durazo-Arvizu RA, et al. Standardizing serum 25-hydroxyvitamin D data from four Nordic population samples using the Vitamin D Standardization Program protocols: Shedding new light on vitamin D status in Nordic individuals. Scand J Clin Lab Invest. 2015;75(7):549-61.
138. Winther A, Dennison E, Ahmed LA, Furberg A-S, Grimnes G, Jorde R, et al. The Tromsø Study: Fit Futures: a study of Norwegian adolescents' lifestyle and bone health. Arch Osteoporos. 2014;9(1):1-11.
139. Jacobsen BK, Eggen AE, Mathiesen EB, Wilsgaard T, Njølstad I. Cohort profile: the Tromsø study. Int J Epidemiol 2011;41(4):961-7.
140. Grimnes G, Almaas B, Eggen AE, Emaus N, Figenschau Y, Hopstock LA, et al. Effect of smoking on the serum levels of 25-hydroxyvitamin D depends on the assay employed. Eur J Endocrinol. 2010;163(2):339-48.
141. van Dam RM, Snijder MB, Dekker JM, Stehouwer CD, Bouter LM, Heine RJ, et al. Potentially modifiable determinants of vitamin D status in an older population in the Netherlands: the Hoorn Study. Am J Clin Nutr. 2007;85(3):755-61.
142. Huisman M, Poppelaars J, van der Horst M, Beekman AT, Brug J, van Tilburg TG, et al. Cohort profile: the longitudinal aging study Amsterdam. Int J Epidemiol. 2011;40(4):dyq219.
143. Moschonis G, Tanagra S, Vandorou A, Kyriakou AE, Dede V, Siatitsa PE, et al. Social, economic and demographic correlates of overweight and obesity in primary-school children: preliminary data from the Healthy Growth Study. Public Health Nutr. 2010;13(10):1693.
144. Siddiqui AM, Kamfar HZ. Prevalence of vitamin D deficiency rickets in adolescent school girls in Western region, Saudi Arabia. Saudi Med J. 2007;28(3):441-4.
145. Al-Othman A, Al-Musharaf S, Al-Daghri NM, Krishnaswamy S, Yusuf DS, Alkharfy KM, et al. Effect of physical activity and sun exposure on vitamin D status of Saudi children and adolescents. BMC pediatr. 2012;12(1):1.
146. Al-Daghri NM, Al-Saleh Y, Khan N, Sabico S, Aljohani N, Alfawaz H, et al. Sun exposure, skin color and vitamin D status in Arab children and adults. J Steroid Biochem Mol Biol. 2016;164:235.
147. Al Faraj S, Al Mutairi K. Vitamin D deficiency and chronic low back pain in Saudi Arabia. Spine. 2003;28(2):177-9.
148. Alfawaz H, Tamim H, Alharbi S, Aljaser S, Tamimi W. Vitamin D status among patients visiting a tertiary care center in Riyadh, Saudi Arabia: a retrospective review of 3475 cases. BMC public health. 2014;14(1):1.
149. Ardawi M-S, Sibiany A, Bakhsh T, Qari M, Maimani A. High prevalence of vitamin D deficiency among healthy Saudi Arabian men: relationship to bone mineral density, parathyroid hormone, bone turnover markers, and lifestyle factors. Osteoporos Int. 2012;23(2):675-86.
150. Moussavi M, Heidarpour R, Aminorroaya A, Pournaghshband Z, Amini M. Prevalence of vitamin D deficiency in Isfahani high school students in 2004. Horm Res. 2005;64(3):144-8.
151. Fuleihan GE-H, Nabulsi M, Choucair M, Salamoun M, Shahine CH, Kizirian A, et al. Hypovitaminosis D in healthy schoolchildren. Pediatrics. 2001;107(4):e53-e.

152. El-Hajj Fuleihan G, Nabulsi M, Tamim H, Maalouf J, Salamoun M, Khalife H, et al. Effect of vitamin D replacement on musculoskeletal parameters in school children: a randomized controlled trial. J Clin Endocrinol Metab. 2006;91(2):405-12.
153. Vogel RA. Measurement of Endothelial Function by Brachial Artery Flow–Mediated Vasodilation. Am J Cardiol. 2001;88(2A):31E-4E.
154. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction. Circulation. 2007;115(10):1285-95.
155. Bonetti PO, Barsness GW, Keelan PC, Schnell TI, Pumper GM, Kuvin JT, et al. Enhanced external counterpulsation improves endothelial function in patients with symptomatic coronary artery disease. J Am Coll Cardiol. 2003;41(10):1761-8.
156. Hashimoto M, Miyamoto Y, Matsuda Y, Akita H. New methods to evaluate endothelial function: non-invasive method of evaluating endothelial function in humans. J Pharmacol Sci. 2003;93(4):405-8.
157. Bonetti PO, Pumper GM, Higano ST, Holmes DR, Kuvin JT, Lerman A. Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. J Am Coll Cardiol. 2004;44(11):2137-41.
158. Haller MJ, Stein J, Shuster J, Theriaque D, Silverstein J, Schatz DA, et al. Peripheral artery tonometry demonstrates altered endothelial function in children with type 1 diabetes. Pediatr Diabetes. 2007;8(4):193-8.
159. Chen C-H, Ting C-T, Nussbacher A, Nevo E, Kass DA, Pak P, et al. Validation of carotid artery tonometry as a means of estimating augmentation index of ascending aortic pressure. Hypertension. 1996;27(2):168-75.
160. Shimizu M, Kario K. Review: Role of the augmentation index in hypertension. Ther Adv Cardiovasc Dis. 2008;2(1):25-35.
161. Takazawa K, Kobayashi H, Shindo N, Tanaka N, Yamashina A. Relationship between radial and central arterial pulse wave and evaluation of central aortic pressure using the radial arterial pulse wave. Hypertens Res. 2007;30(3):219-28.
162. Gurovich AN, Braith RW. Pulse wave analysis and pulse wave velocity techniques: are they ready for the clinic? Hypertens Res. 2011;34(2):166.
163. McEniery CM, Qasem A, Schmitt M, Avolio AP, Cockcroft JR, Wilkinson IB. Endothelin-1 regulates arterial pulse wave velocity in vivo. J Am Coll Cardiol. 2003;42(11):1975-81.
164. Donald AE, Charakida M, Cole TJ, Friberg P, Chowienczyk PJ, Millasseau SC, et al. Non-invasive assessment of endothelial function. J Am Coll Cardiol. 2006;48(9):1846-50.
165. Mlinar B, Marc J, Janez A, Pfeifer M. Molecular mechanisms of insulin resistance and associated diseases. Clin Chim Acta. 2007;375(1-2):20-35.
166. Vane JR, Anggard EE, Botting RM. Regulatory functions of the vascular endothelium. N Engl J Med. 1990;323:27–36.
167. C Brewer L, D Michos E, P Reis J. Vitamin D in atherosclerosis, vascular disease, and endothelial function. Curr Drug Targets. 2011;12(1):54-60.
168. Ahmad S, Hewett PW, Wang P, Al-Ani B, Cudmore M, Fujisawa T, et al. Direct evidence for endothelial vascular endothelial growth factor receptor-1 function in nitric oxide–mediated angiogenesis. Circ Res. 2006;99(7):715-22.
169. Ni W, Watts SW, Ng M, Chen S, Glenn DJ, Gardner DG. Elimination of vitamin D receptor in vascular endothelial cells alters vascular function. Hypertension. 2014;64(6):1290-8.
170. Yiu Y-F, Chan Y-H, Yiu K-H, Siu C-W, Li S-W, Wong L-Y, et al. Vitamin D deficiency is associated with depletion of circulating endothelial progenitor cells and endothelial dysfunction in patients with type 2 diabetes. J Clin Endocrinol Metab. 2011;96(5):E830-E5.
171. Sugden J, Davies J, Witham M, Morris A, Struthers A. Vitamin D improves endothelial function in patients with Type 2 diabetes mellitus and low vitamin D levels. Diabet Med. 2008;25(3):320-5.
172. Stojanović M, Radenković M. Vitamin D Versus Placebo in Improvement of Endothelial Dysfunction: A Meta-Analysis of Randomized Clinical Trials. Cardiovasc Ther. 2015;33(3):145-54.

173. Yiu Y-F, Yiu K-H, Siu C-W, Chan Y-H, Li S-W, Wong L-Y, et al. Randomized controlled trial of vitamin D supplement on endothelial function in patients with type 2 diabetes. Atherosclerosis. 2013;227(1):140-6.
174. Witham MD, Price RJG, Struthers AD, Donnan PT, Messow CM, Ford I, et al. Cholecalciferol treatment to reduce blood pressure in older patients with isolated systolic hypertension the VitDISH randomized controlled trial. JAMA Intern Med. 2013b;173(18):1672-9.
175. Witham MD, Dove FJ, Khan F, Lang CC, Belch JJF, Struthers AD. Effects of Vitamin D supplementation on markers of vascular function after myocardial infarction - A randomised controlled trial. Int J Cardiol. 2013a;167(3):745-9.
176. Witham M, Dove F, Sugden J, Doney A, Struthers A. The effect of vitamin D replacement on markers of vascular health in stroke patients–A randomised controlled trial. Nutr Metab Cardiovasc Dis. 2012;22(10):864-70.
177. Witham MD, Dove FJ, Dryburgh M, Sugden JA, Morris AD, Struthers AD. The effect of different doses of vitamin D3 on markers of vascular health in patients with type 2 diabetes: A randomised controlled trial. Diabetologia. 2010;53(10):2112-9.
178. Stricker H, Bianda FT, Guidicelli-Nicolosi S, Limoni C, Colucci G. Effect of a single, oral, high-dose vitamin D supplementation on endothelial function in patients with peripheral arterial disease: a randomised controlled pilot study. Eur J Vasc Endovasc Surg. 2012;44(3):307-12.
179. Sokol SI, Srinivas V, Crandall JP, Kim M, Tellides G, Lebastchi A, et al. The effects of vitamin D repletion on endothelial function and inflammation in patients with coronary artery disease. Vasc Med. 2012;17(6):394-404.
180. Marckmann P, Agerskov H, Thineshkumar S, Bladbjerg E-M, Sidelmann JJ, Jespersen J, et al. Randomized controlled trial of cholecalciferol supplementation in chronic kidney disease patients with hypovitaminosis D. Nephrol Dial Transplant. 2012;27(9):3523-31.
181. Larsen T, Mose FH, Bech JN, Hansen AB, Pedersen EB. Effect of cholecalciferol supplementation during winter months in patients with hypertension: a randomized, placebo-controlled trial. Am J Hypertens. 2012;25(11):1215-22.
182. Hewitt NA, O'Connor AA, O'Shaughnessy DV, Elder GJ. Effects of cholecalciferol on functional, biochemical, vascular, and quality of life outcomes in hemodialysis patients. Clin J Am Soc Nephrol. 2013;8(7):1143-9.
183. Gepner AD, Ramamurthy R, Krueger DC, Korcarz CE, Binkley N, Stein JH. A prospective randomized controlled trial of the effects of vitamin D supplementation on cardiovascular disease risk. PloS one. 2012;7(5):e36617.
184. Chagas CEA, Borges MC, Martini LA, Rogero MM. Focus on vitamin D, inflammation and type 2 diabetes. Nutrients. 2012;4(1):52-67.
185. Sigmundsdottir H, Pan J, Debes GF, Alt C, Habtezion A, Soler D, et al. DCs metabolize sunlight-induced vitamin D3 to'program'T cell attraction to the epidermal chemokine CCL27. Nat Immunol. 2007;8(3):285-93.
186. Adamczak D, Nowak J, Frydrychowicz M, Kaczmarek M, Sikora J. The Role of Toll-Like Receptors and Vitamin D in Diabetes Mellitus Type 1–A Review. Scand J Immunol. 2014;80(2):75-84.
187. Góral A, Brola W, Kasprzyk M, Przybylski W. The role of vitamin D in the pathogenesis and course of multiple sclerosis. Wiad Lek. 2014;68(1):60-6.
188. Wang J, Lv S, Chen G, Gao C, He J, Zhong H, et al. Meta-analysis of the association between vitamin D and autoimmune thyroid disease. Nutrients. 2015;7(4):2485-98.
189. Facchini L, Venturini E, Galli L, Martino Md, Chiappini E. Vitamin D and tuberculosis: a review on a hot topic. J Chemother. 2015;27(3):128-38.
190. Korf H, Decallonne B, Mathieu C. Vitamin D for infections. Curr Opin Endocrinol Diabetes Obes. 2014;21(6):431-6.
191. Phillips KM, Byrdwell WC, Exler J, Harnly JM, Holden JM, Holick MF, et al. Development and validation of control materials for the measurement of vitamin D 3 in selected US foods. J Food Compost Anal. 2008;21(7):527-34.

192. Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, et al. Role of the Toll-like receptor 4/NF-κB pathway in saturated fatty acid–induced inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol. 2007;27(1):84-91.
193. Higashi Y, Noma K, Yoshizumi M, Kihara Y. Endothelial function and oxidative stress in cardiovascular diseases. Circ J. 2009;73(3):411-8.
194. Soares MJ, Pathak K, Calton EK. Calcium and vitamin D in the regulation of energy balance: where do we stand? Int J Mol Sci. 2014;15(3):4938-45.
195. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. Am J Clin Nutr. 2008;87(4):1080S-6S.
196. Peterlik M, Boonen S, Cross HS, Lamberg-Allardt C. Vitamin D and calcium insufficiency-related chronic diseases: an emerging world-wide public health problem. Int J Environ Res Public Health. 2009;6(10):2585-607.
197. Heaney RP. Vitamin D in health and disease. Clin J Am Soci Nephrol 2008;3(5):1535-41.
198. Meigs JB, Hu FB, Rifai N, Manson JE. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. JAMA. 2004;291(16):1978-86.
199. Flavahan N. Atherosclerosis or lipoprotein-induced endothelial dysfunction. Potential mechanisms underlying reduction in EDRF/nitric oxide activity. Circulation. 1992;85(5):1927-38.
200. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res. 2000;87(10):840-4.
201. Boos CJ, Lip GY. Is hypertension an inflammatory process? Curr pharm des. 2006;12(13):1623-35.
202. Alvarez JA, Ashraf A. Role of vitamin D in insulin secretion and insulin sensitivity for glucose homeostasis. Int J Endocrinol. 2010;2010.
203. Andrukhova O, Slavic S, Zeitz U, Riesen SC, Heppelmann MS, Ambrisko TD, et al. Vitamin D is a regulator of endothelial nitric oxide synthase and arterial stiffness in mice. Mol Endocrinol. 2013;28(1):53-64.
204. Liu S, Song Y, Ford ES, Manson JE, Buring JE, Ridker PM. Dietary calcium, vitamin D, and the prevalence of metabolic syndrome in middle-aged and older US women. Diabetes Care. 2005;28(12):2926-32.
205. Pittas AG, Dawson-Hughes B, Li T, Van Dam RM, Willett WC, Manson JE, et al. Vitamin D and calcium intake in relation to type 2 diabetes in women. Diabetes Care. 2006;29(3):650-6.
206. Knekt P, Laaksonen M, Mattila C, Härkänen T, Marniemi J, Heliövaara M, et al. Serum vitamin D and subsequent occurrence of type 2 diabetes. Epidemiology. 2008;19(5):666-71.
207. Soares M, Kuriyan R, Kurpad A. Calcium and vitamin D modulate postprandial vascular function: A pilot dose–response study. Diabetes Metab Syndr: Clin Res Rev. 2010;4(3):128-31.
208. Alyami AM, Lam V, Soares MJ, Zhao Y, Sherriff JL, Mamo JC, et al. The association of vitamin D status with dyslipidaemia and biomarkers of endothelial cell activation in older Australians. Nutrients. 2016;8(8):457.
209. Bajaj A, Stone KL, Peters K, Parimi N, Barrett-Connor E, Bauer D, et al. Circulating vitamin D, supplement use, and cardiovascular disease risk: the MrOS sleep study. J Clin Endocrinol Metab. 2014;99(9):3256-62.
210. Sollid ST, Hutchinson MY, Fuskevåg OM, Figenschau Y, Joakimsen RM, Schirmer H, et al. No effect of high-dose vitamin D supplementation on glycemic status or cardiovascular risk factors in subjects with prediabetes. Diabetes Care. 2014;37(8):2123-31.
211. Breslavsky A, Frand J, Maras Z, Boaz M, Barnea Z, Shargorodsky M. Effect of high doses of vitamin D on arterial properties, adiponectin, leptin and glucose homeostasis in type 2 diabetic patients. Clin Nutr. 2013;32(6):970-5.
212. Harris RA, Pedersen-White J, Guo D-H, Stallmann-Jorgensen IS, Keeton D, Huang Y, et al. Vitamin D3 supplementation for 16 weeks improves flow-mediated dilation in overweight African-American adults. Am J Hypertens. 2011;24(5):557-62.
213. Wang L, Manson JE, Song Y, Sesso HD. Systematic review: vitamin D and calcium supplementation in prevention of cardiovascular events. Ann Intern Med. 2010;152(5):315-23.

214. Chung M, Tang AM, Fu Z, Wang DD, Newberry SJ. Calcium Intake and Cardiovascular Disease RiskAn Updated Systematic Review and Meta-analysisCalcium Intake and Cardiovascular Disease Risk. Ann Intern Med. 2016;165(12):856-66.
215. Bolland MJ, Barber PA, Doughty RN, Mason B, Horne A, Ames R, et al. Vascular events in healthy older women receiving calcium supplementation: randomised controlled trial. BMJ. 2008;336(7638):262-6.
216. Pittas AG, Lau J, Hu FB, Dawson-Hughes B. The role of vitamin D and calcium in type 2 diabetes. A systematic review and meta-analysis. J Clin Endocrinol Metab. 2007;92(6):2017-29.
217. Mitri J, Dawson-Hughes B, Hu FB, Pittas AG. Effects of vitamin D and calcium supplementation on pancreatic β cell function, insulin sensitivity, and glycemia in adults at high risk of diabetes: the Calcium and Vitamin D for Diabetes Mellitus (CaDDM) randomized controlled trial. Am J Clin Nutr. 2011;94(2):486-94.
218. Pittas AG, Harris SS, Stark PC, Dawson-Hughes B. The effects of calcium and vitamin D supplementation on blood glucose and markers of inflammation in nondiabetic adults. Diabetes care. 2007;30(4):980-6.
219. De Boer IH, Tinker LF, Connelly S, Curb JD, Howard BV, Kestenbaum B, et al. Calcium plus vitamin D supplementation and the risk of incident diabetes in the Women's Health Initiative. Diabetes care. 2008;31(4):701-7.
220. Gagnon C, Lu ZX, Magliano DJ, Dunstan DW, Shaw JE, Zimmet PZ, et al. Serum 25-hydroxyvitamin D, calcium intake, and risk of type 2 diabetes after 5 years. Diabetes care. 2011;34(5):1133-8.
221. Nikooyeh B, Neyestani TR, Farvid M, Alavi-Majd H, Houshiarrad A, Kalayi A, et al. Daily consumption of vitamin D–or vitamin D+ calcium–fortified yogurt drink improved glycemic control in patients with type 2 diabetes: a randomized clinical trial. Am J Clin Nutr. 2011;93(4):764-71.
222. Hussin AM, Ashor AW, Schoenmakers I, Hill T, Mathers JC, Siervo M. Effects of vitamin D supplementation on endothelial function: a systematic review and meta-analysis of randomised clinical trials. Eur J Nutr. 2016;Epub ahead of print:1-10.
223. Moerland M, Kales A, Schrier L, Van Dongen M, Bradnock D, Burggraaf J. Evaluation of the EndoPAT as a tool to assess endothelial function. Int J Vasc Med. 2012;2012(Article ID 904141):8 pages.
224. Alyami A, Soares M, Sherriff J, Zhao Y, Hallett J, Coombes F. A systematic review protocol examining the effect of vitamin D supplementation on endothelial function. BMJ open. 2015;5(6):e006835.
225. Centre for Evidence in Transplantation. Assessment of Randomised Controlled Trials (RCTs) 2010 [updated 2014. Available from: http://www.transplantevidence.com/library_rct.php.
226. Jadad AR, Moore RA, Carroll D, Jenkinson C, Reynolds DJM, Gavaghan DJ, et al. Assessing the quality of reports of randomized clinical trials: is blinding necessary? Control Clin Trials. 1996;17(1):1-12.
227. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Int J Surg. 2010;8(5):336-41.
228. Muldowney S, Lucey AJ, Hill TR, Seamans KM, Taylor N, Wallace JM, et al. Incremental cholecalciferol supplementation up to 15 μg/d throughout winter at 51–55 N has no effect on biomarkers of cardiovascular risk in healthy young and older adults. J Nutr. 2012;142(8):1519-25.
229. Wood AD, Secombes KR, Thies F, Aucott L, Black AJ, Mavroeidi A, et al. Vitamin D 3 supplementation has no effect on conventional cardiovascular risk factors: A parallel-group, double-blind, placebo-controlled RCT. J Clin Endocrinol Metab. 2012;97(10):3557-67.
230. Ryu OH, Chung W, Lee S, Hong KS, Choi MG, Yoo HJ. The effect of high-dose vitamin D supplementation on insulin resistance and arterial stiffness in patients with type 2 diabetes. Korean J Intern Med. 2014;29(5):620-9.
231. Sinha-Hikim I, Duran P, Shen R, Lee M, Friedman TC, Davidson MB. Effect of Long Term Vitamin D Supplementation on Biomarkers of Inflammation in Latino and African-American Subjects with Pre-Diabetes and Hypovitaminosis D. Horm Metab Res. 2014;47:280-3.

232. Gagnon C, Daly RM, Carpentier A, Lu ZX, Shore-Lorenti C, Sikaris K, et al. Effects of Combined Calcium and Vitamin D Supplementation on Insulin Secretion, Insulin Sensitivity and beta-Cell Function in Multi-Ethnic Vitamin D-Deficient Adults at Risk for Type 2 Diabetes: A Pilot Randomized, Placebo-Controlled Trial. Plos One. 2014;9(10).
233. Forouhi N, Menon R, Sharp S, Mannan N, Timms P, Martineau A, et al. Effects of vitamin D2 or D3 supplementation on glycaemic control and cardiometabolic risk among people at risk of type 2 diabetes: results of a randomized double-blind placebo-controlled trial. Diabetes Obes Metab. 2016;18(4).
234. Wamberg L, Kampmann U, Stodkilde-Jorgensen H, Rejnmark L, Pedersen SB, Richelsen B. Effects of vitamin D supplementation on body fat accumulation, inflammation, and metabolic risk factors in obese adults with low vitamin D levels - Results from a randomized trial. Euro J Intern Med. 2013;24(7):644-9.
235. Adeyemo A, Gerry N, Chen G, Herbert A, Doumatey A, Huang H, et al. A genome-wide association study of hypertension and blood pressure in African Americans. PLoS Genet. 2009;5(7):e1000564.
236. Pannu PK, Zhao Y, Soares MJ. Reductions in body weight and percent fat mass increase the vitamin D status of obese subjects: a systematic review and metaregression analysis. Nutr Res. 2016;36(3):201-13.
237. Nowson CA, Margerison C. Vitamin D intake and vitamin D status of Australians. Med J Aust. 2002;177(3):149-52.
238. Sesso HD, Buring JE, Rifai N, Blake GJ, Gaziano JM, Ridker PM. C-reactive protein and the risk of developing hypertension. JAMA. 2003;290(22):2945-51.
239. Page AV, Liles WC. Biomarkers of endothelial activation/dysfunction in infectious diseases. Virulence. 2013;4(6):507-16.
240. Sibal L, Aldibbiat A, Agarwal S, Mitchell G, Oates C, Razvi S, et al. Circulating endothelial progenitor cells, endothelial function, carotid intima–media thickness and circulating markers of endothelial dysfunction in people with type 1 diabetes without macrovascular disease or microalbuminuria. Diabetologia. 2009;52(8):1464-73.
241. Kathiresan S, Gona P, Larson MG, Vita JA, Mitchell GF, Tofler GH, et al. Cross-sectional relations of multiple biomarkers from distinct biological pathways to brachial artery endothelial function. Circulation. 2006;113(7):938-45.
242. Atuma S, Aune M. Method for the determination of PCB congeners and chlorinated pesticides in human blood serum. Bull Environ Contam Toxicol. 1999;62(1):8-15.
243. USDA. Dietary Guidelines for Americans. Department of Health and Human Services; 1990.
244. Norton K, Olds T. Anthropometrica: a textbook of body measurement for sports and health courses. Australia: UNSW press; 1996.
245. Alberti K, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome a joint interim statement of the international diabetes federation task force on epidemiology and prevention; national heart, lung, and blood institute; American heart association; world heart federation; international atherosclerosis society; and international association for the study of obesity. Circulation. 2009;120(16):1640-5.
246. Lamounier-Zepter V, Ehrhart-Bornstein M, Bornstein SR. Insulin resistance in hypertension and cardiovascular disease. Best Pract Res Clin Endocrinol Metab. 2006;20(3):355-67.
247. Colman P, Thomas D, Zimmet P, Welborn T, Garcia-Webb P, Moore M. New classification and criteria for diagnosis of diabetes mellitus. The Australasian Working Party on Diagnostic Criteria for Diabetes Mellitus. N Z Med J. 1999;112(1086):139-41.
248. Lorenzo C, Haffner SM, Stancakova A, Laakso M. Relation of direct and surrogate measures of insulin resistance to cardiovascular risk factors in nondiabetic Finnish offspring of type 2 diabetic individuals. J Clin Endocrinol Metab. 2010;95(11):5082-90.
249. Sluiter W, Erkelens D, Reitsma W, Doorenbos H. Glucose tolerance and insulin release, a mathematical approach: I. Assay of the beta-cell response after oral glucose loading. Diabetes. 1976;25(4):241-4.

250. Matthews D, Hosker J, Rudenski A, Naylor B, Treacher D, Turner R. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28(7):412-9.
251. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab. 2000;85(7):2402-10.
252. McAuley KA, Williams SM, Mann JI, Walker RJ, Lewis-Barned NJ, Temple LA, et al. Diagnosing insulin resistance in the general population. Diabetes Care. 2001;24(3):460-4.
253. Chowienczyk PJ, Kelly RP, MacCallum H, Millasseau SC, Andersson TL, Gosling RG, et al. Photoplethysmographic assessment of pulse wave reflection: blunted response to endothelium-dependent beta2-adrenergic vasodilation in type II diabetes mellitus. J Am Coll Cardiol. 1999;34(7):2007-14.
254. Millasseau S, Kelly R, Ritter J, Chowienczyk P. Determination of age-related increases in large artery stiffness by digital pulse contour analysis. Clin Sci (Lond). 2002;103(4):371-7.
255. Woodman RJ, Watts GF, Kingwell BA, Dart A. Interpretation of the digital volume pulse; its relationship with large and small artery compliance. Clin Sci (Lond). 2003;104:283-5.
256. Chan She Ping-Delfos W PL, Soares MJ. Digital volume pulse and
endothelial function: reproducibility and comparison of instruments. Asia Pac J Clin Nutr 2007;16((Suppl. 3)):S117.
257. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract. 2010;87(1):4-14.
258. Motlagh B, O'Donnell M, Yusuf S. Prevalence of cardiovascular risk factors in the Middle East: a systematic review. Eur J Cardiovasc Prev Rehabil. 2009;16(3):268-80.
259. Bahijri SM, Jambi HA, Al Raddadi RM, Ferns G, Tuomilehto J. The Prevalence of Diabetes and Prediabetes in the Adult Population of Jeddah, Saudi Arabia-A Community-Based Survey. PloS one. 2016;11(4):e0152559.
260. Alzeidan R, Rabiee F, Hersi A, Mandil A. 26. Cardiovascular risk assessment for Saudi university employees and their families: developing a framework for provision of an evidence-based cardiovascular disease preventative programme. J Saudi Heart Ass. 2016;28(3):197-8.
261. Black LJ, Anderson D, Clarke MW, Ponsonby A-L, Lucas RM. Analytical bias in the measurement of serum 25-hydroxyvitamin D concentrations impairs assessment of vitamin D status in clinical and research settings. PloS one. 2015;10(8):e0135478.
262. Millasseau SC, Kelly RP, Ritter JM, Chowienczyk PJ. The vascular impact of aging andvasoactive drugs: comparison of two digital volume pulse measurements. A J Hypertens. 2003;16(6):467-72.
263. Hull H, He Q, Thornton J, Javed F, Allen L, Wang J, et al. iDXA, Prodigy, and DPXL dual-energy X-ray absorptiometry whole-body scans: a cross-calibration study. J Clin Densitom. 2009;12(1):95-102.
264. Avogaro A, Albiero M, Menegazzo L, de Kreutzenberg S, Fadini GP. Endothelial dysfunction in diabetes the role of reparatory mechanisms. Diabetes Care. 2011;34(Supplement 2):S285-S90.
265. Kalinowski L, Dobrucki IT, Malinski T. Race-Specific Differences in Endothelial Function: Predisposition of African Americans to Vascular Diseases. . Circulation. 2004;109(21):2511-17.
266. Marroni AS, Metzger IF, Souza-Costa DC, Nagassaki S, Sandrim VC, Correa RX, et al. Consistent interethnic differences in the distribution of clinically relevant endothelial nitric oxide synthase genetic polymorphisms. Nitric Oxide. 2005;12(3):177-82.
267. Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M. Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. J Clin Invest. 2001;108(9):1341.
268. Borch-Johnsen K, Neil A, Balkau B, Larsen S, Nissinen A, Pekkanen J, et al. Glucose tolerance and cardiovascular mortality-Comparison of fasting and 2-hour diagnostic criteria. 2001.
269. Wopereis S, Wolvers D, van Erk M, Gribnau M, Kremer B, van Dorsten FA, et al. Assessment of inflammatory resilience in healthy subjects using dietary lipid and glucose challenges. BMC medical genomics. 2013;6(1):44.

270. Wiklund P, Toss F, Weinehall L, Hallmans G, Franks PW, Nordstrom A, et al. Abdominal and gynoid fat mass are associated with cardiovascular risk factors in men and women. J Clin Endocrinol Metab. 2008;93(11):4360-6.
271. Lu C-L, Zou X, Orr WC, Chen J. Postprandial changes of sympathovagal balance measured by heart rate variability. Dig Dis Sci. 1999;44(4):857-61.
272. Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M. Vitamin D and DBP: the free hormone hypothesis revisited. J Steroid Biochem Mol Biol. 2014;144:132-7.
273. Bouillon R, Van Assche FA, Van Baelen H, Heyns W, De Moor P. Influence of the vitamin D-binding protein on the serum concentration of 1, 25-dihydroxyvitamin D3: significance of the free 1, 25-dihydroxyvitamin D3 concentration. J Clin Invest. 1981;67(3):589-96.
274. MENDEL CM. The Free Hormone Hypothesis: A Physiologically Based Mathematical Model*. Endocr Rev. 1989;10(3):232-74.
275. Hewison M. An update on vitamin D and human immunity. Clin Endocrinol (Oxf). 2012;76(3):315-25.
276. Lauridsen AL, Vestergaard P, Hermann A, Brot C, Heickendorff L, Mosekilde L, et al. Plasma concentrations of 25-hydroxy-vitamin D and 1, 25-dihydroxy-vitamin D are related to the phenotype of Gc (vitamin D-binding protein): a cross-sectional study on 595 early postmenopausal women. Calcif Tissue Int. 2005;77(1):15-22.
277. Mitchell D, Henao M, Finkelstein J, Burnett-Bowie S-A. Prevalence and predictors of vitamin D deficiency in healthy adults. Endocr Pract. 2012;18(6):914-23.
278. Gutierrez O, Farwell W, Kermah D, Taylor E. Racial differences in the relationship between vitamin D, bone mineral density, and parathyroid hormone in the National Health and Nutrition Examination Survey. Osteoporos Int. 2011;22(6):1745-53.
279. Ginde AA, Liu MC, Camargo CA. Demographic differences and trends of vitamin D insufficiency in the US population, 1988-2004. Arch Intern Med. 2009;169(6):626-32.
280. Henry HL, Bouillon R, Norman AW, Gallagher JC, Lips P, Heaney RP, et al. 14th Vitamin D Workshop consensus on vitamin D nutritional guidelines. J Steroid Biochem Mol Biol. 2010;121(1):4-6.
281. Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Deanfield JE. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. J Am Coll Cardiol. 1994;24(2):471-6.
282. Chang EI, Loh SA, Ceradini DJ, Chang EI, Lin S-e, Bastidas N, et al. Age decreases endothelial progenitor cell recruitment through decreases in hypoxia-inducible factor 1α stabilization during ischemia. Circulation. 2007;116(24):2818-29.
283. Taddei S, Virdis A, Mattei P, Ghiadoni L, Gennari A, Fasolo CB, et al. Aging and endothelial function in normotensive subjects and patients with essential hypertension. Circulation. 1995;91(7):1981-7.
284. Hathcock JN, Shao A, Vieth R, Heaney R. Risk assessment for vitamin D. Am J Clin Nutr 2007;85(1):6-18.
285. Jorde R, Schirmer H, Wilsgaard T, Mathiesen EB, Njølstad I, Løchen M-L, et al. The DBP phenotype Gc-1f/Gc-1f is associated with reduced risk of cancer. The tromsø study. PloS one. 2015;10(5):e0126359.
286. Arnaud J, Constans J. Affinity differences for vitamin D metabolites associated with the genetic isoforms of the human serum carrier protein (DBP). Hum Genet. 1993;92(2):183-8.

Appendices

Appendix 1: Physical activity checklist: (International Physical Activity Questionnaire (IPAQ))

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

_____ Days per week

☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

_____ Hours per day
_____ Minutes per day

☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ Days per week

☐ No moderate physical activities → ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ Hours per day
_____ Minutes per day

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ Days per week

☐ No walking → Skip to question 7

6. How much time did you usually spend **walking** on one of those days?

_____ Hours per day
_____ Minutes per day

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ Hours per day
_____ Minutes per day

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

Appendix 2: Screening Survey

Project Title: Vitamin D and endothelial function

Thank you for your interest in participating in this research. This study has been approved by the Curtin University Human Research Ethics Committee (Approval number:). This Committee consists of academics, doctors, lawyers, members of the public and pastoral carers. If necessary, verification of approval can be acquired through correspondence by post to the Curtin University Human

Research Ethics Committee c/- Office of Research Development, Curtin University of Technology, GPO Box U1987, Perth 6845, by telephone at 9266 2784 or by email to *hrec@curtin.edu.au*.

Purpose of study:

You are invited to participate in a study which will investigate the association between circulating 25(OH)D with endothelial dysfunction. The outcomes will be considered with regards to the results of the preceding literature review. At the conclusion of this study, you will be provided with a report that details your endothelial function data, body composition (fat, muscle and bone mass as well as fat percentage) and also your blood profiles of glucose, insulin, triglycerides, HDL cholesterol, blood pressure and the liver function test.

Screening Survey

To determine if you meet the eligibility criteria for this study, we ask that you kindly fill in this short survey. Alternatively, this form may be completed online. There are no known risks associated with completing this application.

Confidentiality

Your personal information will be solely used for the purpose of this study and will be stored in a protective place even after study completion, to ensure your privacy.
For further information or copies of this survey, please contact Ali Alyami by email:
alimahdim.alyami@student.curtin.edu.au

☐ I have read and understood these study requirements and I am ready to participate.

Please see the survey in the following pages.

Please fill in the form below by drawing an (X) in each box corresponding to your answer. However, more details may be requested upon answering YES to some questions.

1. Demographics

a) First name: _____

a) Last name: _____

b) Address: _____

Suburb: _____

Postcode: _____

c) Telephone: _____ (home)

_____ (mobile)

d) Email _____

e) Date of Birth: _____ Age: _____ years

f) Menopause: Yes ☐
No ☐
In between ☐
Not Applicable ☐

a) Your birth Country: _____

b) Duration lived in Australia: _____ (years)

c) Birth countries of parents: _____ & _____

2. *Do you currently smoke?* ☐ Y ☐ N

3. Do you drink more than 2 standard alcoholic drinks per day? ☐ Y ☐ N

4. Has your weight fluctuated by 2kg (or more) during the last 6 months ☐ Y ☐ N

5. Do you plan to try to lose weight in the next 6 months? ☐ Y ☐ N

6. Are you pregnant or planning to become pregnant in the next 6 months? ☐ Y ☐ N

7. Are you breastfeeding or planning to breastfeed in the next 6 months? ☐ Y ☐ N

8. Do you suffer from any of the following conditions?

a) Diabetes ☐Y ☐N

☐ype 1 ☐Type 2

Diagnosed for _____ years. Is it well controlled? ☐s ☐No ☐/A

Recent HbA1C (within the last 6 months), if known: _____

Please provide further details, if applicable:

b) Do you suffer from any thyroid condition? ☐es ☐No

c) Do you have an elevated blood lipid profile (triglycerides, cholesterol, etc.)?

Y ☐ N ☐

d) Do you have hypertension (high blood pressure)? Y ☐ N ☐

e) Do you have a history of cardiovascular diseases (heart problem requiring hospital care)?
Y ☐ N ☐

f) Kidney problems Y ☐ N ☐

g) Any conditions not mentioned above? Y ☐ N ☐

9. Are you currently taking any of the following medications?

a) Hormone replacement therapy ☐ Y ☐ N

b) Steroids ☐ Y ☐ N

c) Cholesterol controlling drugs Y ☐ N ☐

d) Vitamin D Supplements Y ☐ N ☐

e) Weight management products Y ☐ N ☐

f) Any other medicines or supplements? Y ☐ N ☐
If Yes provide details

10. We will provide all the participants with one of the following meals as part of the preparation for the test. Please select <u>one</u> of the following:

a) Butter chicken and rice (2-minute cooking) ☐
b) Chicken satay and rice (2-minute cooking) ☐
c) Beef black pepper and rice (2-minute cooking) ☐

d) Vegetable curry and rice (2-minute cooking)- Vegetarian ☐

11. Do you have any food allergies or intolerances? Y ☐ N ☐

If Yes, please provide details below:

Thank you for completing this survey.