Does vitamin D deficiency contribute to endothelial dysfunction?

by

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Thesis submitted for the degree of

Doctor of Philosophy

March 2014

School of Pharmacy and Medical Sciences

Division of Health Sciences



University of
South Australia

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Abbreviations

$1,25\text{-}(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$1\text{-}\alpha\text{-}(OH)D_3$	1-α-hydroxyvitamin D_3
$25\text{-}(OH)D_3$	25-hydroxyvitamin D_3
ACE	Angiotensin I-converting enzyme
ACEI	Angiotensin I-converting enzyme inhibitor
ACTRN	Australian New Zealand clinical trial registry number
ADMA	Asymmetric dimethylarginine
ADP	Adenosine di-phosphate
AGEs	Advanced glycation end products
AGE-HAS	AGE-human serum albumin
ALTM	All-laboratory trimmed mean
AMP	Adenosine monophosphate
ARB	Angiotensin receptor blocker
ATP	Adenosine triphosphate
β	Beta; coefficient correlation (linear regression)
BBMI	Brush border myosin I
BHI	Basil Hetzel Institute
BMI	Body Mass Index
BNP	Brain natriuretic peptide
CAC	Coronary artery calcification
CAD	Coronary artery disease
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine ligand
CD36	Cluster of differentiation 36 (fatty acid translocase (FAT))
CD47	Cluster of differentiation 47 (integrin associated protein (IAP))
cGMP	Cyclic guanosine monophosphate
CHF	Congestive heart failure
CI	Confidence interval
CKD	Chronic kidney disease
COX-1	Cyclooxygenase-1

CRF	Chronic renal failure
CRP	C-reactive protein
CV	Coefficient of variance
CVD	Cardiovascular disease
DBP	Vitamin D Binding Protein
DDAH	Dimethylarginine dimethyl amino hydroxylase
DEA/NO	Diethylamine NONOate
DM	Diabetic Mellitus
DNA	Deoxyribonucleic acid
EDRFs	Endothelium-derived relaxing factors
SEM	Standard error of mean
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
eNOS (NOS3)	Endothelial nitric oxide synthase
EPC	Endothelial Progenitor Cells
EPC	Endothelial progenitor cells
ER	Endoplasmic reticulum
FHx	Family history
FMD	Flow Mediated Dilatation
GC	Guanylyl cyclase
GFR	Glomerular filtration rate
Gla	γ-carboxyglutamic acid
HbA_1C	Glycated hemoglobin / glycosylated hemoglobin
HDL	High density lipoprotein
HF	Heart failure
HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
HOMA	Homeostatic model assessment
HOMA-IR	Homeostatic model assessment-insulin resistance
HPLC	High-performance liquid chromatography / high-pressure liquid chromatography
hs-CRP	High Sensitive C-Reactive Protein
HTN	Hypertension
HUVEC	Human umbilical vein endothelial cells

IDDM	Insulin Dependent Diabetic Mellitus
IDS	Immunodiagnostic system
IDS-EIA	Immunodiagnostic system-Enzyme immunoassay
IFN-γ	Interferon-γ
IHC	Immunohistrochemistry
IL	Interleukin
IMVS	Institute of Medical and Veterinary Science
IR	Insulin resistance
IU	International Unit
LC-MS/MS	Liquid chromatography-mass spectroscopy
LDL	Low density lipoprotein
L-NMMA	N-monomethyl-l-arginine
LoD	Limit of detection (assay limit of detection)
LPS	Lipopolysaccharide
LVESD	Left ventricular end-systolic diameter
MBA20	Multiple Biochemical Analysis 20
MDA	Malondialdehyde
MGP	Matrix Gla protein
MI	Myocardial infarction
mmHg	Millimeter of mercury
µg	Micrograms
µmol	Micromole
min	Minute / minutes
MMP9	Matrix metallopeptidase
mRNA	Messenger Ribonucleic acid
ng	Nanogram
NADPH	Nicotinamide adenine dinucleotide phosphate
NF κB	Nuclear factor κB
NH_2	Amino functional group
NHANES	The Third National Health and Nutrition Examination Survey
NIDDM	Non-insulin dependent diabetic mellitus
NO	Nitric Oxide
NOD	Non-obese diabetic
NOX	Nitric oxide metabolites

NOS	Nitric Oxide Synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
N.S.	Not significant
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
NYHA	New York heart association
O_2^-	Superoxide radical
PAD	Peripheral arterial disease
PO_4^{3-}	Phosphate ion
PGI_2	Prostacyclin
PHPT	Primary hyperparathyroidism
PKC	Protein kinase C
PSP	Parathyroid secretary protein
PTH	Parathyroid hormone
PTX	Parathyroidectomy
PWV	Pulse wave velocity
QC	Quality control
QEH	Queen Elizabeth Hospital
QUICKI	Quantitative insulin-sensitivity check index
RAAS	renin-angiotensin-aldosteron system
RAGE	Receptors for Advanced Glycation End Products
RIA	Radio immunoassay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Randomized trial
RXR	Retinoid X Receptor
SA	South Australia
SBP	Systolic blood pressure
SD	Standard deviation
SDMA	Symmetric dimethylarginine / N,N-dimethyl-l-arginine
sGC	Soluble guanylate cyclase
SH	Sulfur-hydrogen bond
SOD	Superoxide dismutase

SPE	Solid phase extraction
SNP	Sodium nitroprusside
TAS	Total oxidant status
TBARs	Thiobarbituric acid reactants
T2DM	Type-2 diabetic mellitus
TG	Triglycerides
TGF-β1	Transforming-growth factor-β1
TIA	Transient ischaemic attack
TNFα	Tumor necrosis factor alpha
TQEH	The Queen Elizabeth hospital
TSP-1	Thrombospondin-1
TXNIP	Thioredoxin Interacting Protein
TYROBP	Tyrosine kinase binding protein
UV	Ultraviolet
UVB	UV B radiation
UVR	UV Radiation
VDR	Vitamin D Receptor
VDREs	Vitamin D-Responsive Elements
VDRKO	Vitamin D Receptor knock-out
VSMCs	Vascular smooth muscle cells
WHI	Women's health initiative

Abstract

Introduction: Vitamin D deficiency is a rising pandemic worldwide and has been associated with various health consequences. Vitamin D receptor (VDR) is widely expressed with broad spectrum of physiological activities of vitamin D. A growing body of evidence suggests that vitamin D is important in cardiovascular homeostasis and conversely, that vitamin D deficiency may represent a risk factor for the development of coronary artery diseases. Low serum vitamin D levels are associated with elevated parathyroid hormone (PTH) levels. Vitamin D deficiency and secondary hyperparathyroidism, either in combination or independently, have been associated with increased cardiovascular (CV) events. However, mechanistic insights of these findings remain largely elusive. Elevated oxidative stress, inflammatory activation, impaired endothelial function and insulin resistance have been described by previous studies as possible mechanisms for the adverse cardiovascular outcomes in low vitamin D and elevated parathyroid hormone status. Our group has previously demonstrated that low 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentrations are associated with increased concentrations of asymmetric dimethylarginine (ADMA), an endothelial nitric oxide synthase (eNOS) inhibitor and biomarker of increased CV morbidity and mortality in healthy ageing individuals. This relationship persisted even after adjustments for season, age, body mass index (BMI) and other cardiovascular risk factors. However, it was not determined whether this relationship is independent of PTH levels.

Furthermore, Type 2 diabetes mellitus (T2DM) and obesity are associated with elevated levels of ADMA. Equally, obesity and Type 2 diabetes mellitus are associated with an increased prevalence of vitamin D deficiency and increased risk of adverse cardiovascular events. Therefore, it is warranted to assess whether this newly establish relationship between vitamin D levels and ADMA concentrations is present in this high risk cohort of obese diabetics and whether this relationship is independent of PTH levels. The beneficial effects of vitamin D supplementation on vascular endothelial function have also been demonstrated by various studies. Currently, the outcomes of vitamin D supplementation on plasma ADMA concentrations have not been evaluated. Thus, it is further warranted to evaluate whether the correction of vitamin D levels with vitamin D supplementation in vitamin D insufficient but otherwise healthy subjects (who are free from other cardiovascular risk factors) affects plasma ADMA concentrations and whether the change in PTH levels correlates with the change in ADMA concentrations in this cohort. As secondary end-points, we assessed the

relationship between vitamin D and plasma thrombospondin-1 (TSP-1) (another potent inhibitor of the nitric oxide-cyclic GMP (NO-cGMP) signaling pathway in vascular cells and contributes to vascular endothelial dysfunction) concentrations in healthy volunteers and the effects of vitamin D supplementation on plasma TSP-1 levels and other markers of nitric oxide (NO) signaling in subjects with low vitamin D levels.

Aims: This thesis will examine the following: 1) the relationship between ADMA and $25(OH)D_3$ in patients with diabetes and obesity in relation to PTH status, 2) in vitamin D insufficient, but otherwise healthy individuals: whether vitamin D supplementation decreases ADMA concentrations, and 3) the effects of low vitamin D levels and the restoration of vitamin D levels on an other inhibitor of nitric oxide signaling, TSP-1.

Methods: This thesis focuses on three main aspects of vitamin D and endothelial function. To assess the relationship between ADMA and $25(OH)D_3$ in patients with diabetes and obesity, in relation to PTH status, obese (body mass index (BMI) 36.9±6.8 (30.21-64.71) Kg/m^2) diabetic patients (n=164) with normal kidney/liver function, not on vitamin D supplementation and aged between 35-88 years (mean±SD 64.8±11.2 years) were recruited from out-patient clinics of the Queen Elizabeth hospital (TQEH). Their biochemical parameters including plasma concentrations of $25(OH)D_3$ (marker of vitamin D status), ADMA, PTH, high-sensitive C-reactive protein (hs-CRP) (marker of inflammation), glycosylated hemoglobin (HbA_1C) (marker of glycaemic control), plasma electrolyte levels, renal and liver function were assessed.

To determine whether vitamin D supplementation decreases ADMA concentration and to assess the effects of changes in PTH levels on ADMA concentrations, n=35 healthy working adults (age 45±10.8 years) (BMI 28.1±5.6 Kg/m^2) (mean±SD), without any pre-existing CV disease or diabetes and with vitamin D insufficiency (25-hydroxyvitamin D_3 levels ($25(OH)D_3$) < 75 nmol/L (30 ng/mL)) were recruited. Vitamin D supplementation of 2000 IU/day was given to all subjects for 12 weeks. At the baseline and follow-up visits biochemical parameters including plasma concentrations of $25(OH)D_3$, ADMA, PTH, hs-CRP, fasting glucose and insulin, homeostatic model assessment (HOMA) index, lipid profiles, plasma electrolyte levels, renal function, liver function and physiological parameters including blood pressure and platelet NO responsiveness were assessed.

To determine the effects of vitamin D restoration on an another inhibitor of nitric oxide signaling, TSP-1, the ELISA method for assaying TSP-1 was conducted on n=31 healthy

subjects in this cohort (n=4 subject's blood out of total 35 samples collected was heamolyzed and could not be taken for the analysis). To assess the effects of low vitamin D levels on TSP-1, a separate cohort of healthy volunteers (n=58) aged 23 to 71 years and who were not on vitamin D supplementation were recruited. The above biochemical and physiological parameters were assessed for all the subjects.

Results: The key findings of the study were:

1. The ADMA concentrations of the obese diabetic cohort were not correlated with plasma vitamin D_3 ($25(OH)D_3$) levels (β=0.061, p=0.46).

2. PTH level is positively associated with ADMA concentration (β=0.278, p=0.003) independent of vitamin D status, age, BMI and other CV risk factors in obese diabetic patients. The other determinant of ADMA concentration is the level of HbA_1C in these obese-diabetics (β=0.248, p=0.007).

3. Restoration of vitamin D levels by a 12-week vitamin D supplementation program in healthy subjects free from cardiovascular risk factors and who were vitamin D insufficient ($25(OH)D_3$ < 75 nmol/L), did not affect their plasma ADMA concentrations (baseline, 0.56±0.09 µmol/L to follow-up, 0.57±0.08 µmol/L, p=59), while changes of PTH levels with the vitamin D treatment positively correlated with changes of ADMA concentrations (β=0.347, p=0.041).

4. Plasma TSP-1 levels vary inversely with plasma vitamin D ($25(OH)D_3$) levels in healthy adults (β=-0.282, p=0.047) and vitamin D supplementation in the vitamin D-insufficient, but otherwise healthy, individuals leads to a significant reduction in plasma TSP-1 levels (baseline, 592.0±459.0 ng/ml to follow-up 206.7±204.5 ng/ml, p<0.001).

Conclusions: The studies conducted both in healthy subjects and obese diabetic patients confirmed that low vitamin D levels and high PTH levels could be cardiovascular risk factors and the correction of low vitamin D levels could minimize the cardiovascular risk from altered vitamin D and PTH levels. Further studies should evaluate the underlying mechanisms of vitamin D and PTH on ADMA and TSP-1 metabolism.

Declaration

I declare that this thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university.

To the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.



Anjalee Thanuja Amarasekera

March 2014

Acknowledgments

The completion of my dissertation and subsequent PhD. has been a long journey, certainly challenging and rewarding. I could not have succeeded without the invaluable help of several supportive people.

I would like to give my heartfelt gratitude, beginning with Prof. Michael S. Roberts, my principal supervisor. I would like to thanks Prof. Roberts for his support and encouragement given to me throughout my candidature.

Next, I would like to give a heartfelt, special thanks to my co-supervisor, Dr. Doan Ngo. I undertook her project on vitamin D effects on endothelial function of obese-diabetic patients. Dr. Ngo's experience and knowledge on clinical research have influenced me to construct a foundation on my project. She has always been a tremendous help to me no matter the task or the circumstance. Working under her supervision has been a great learning experience which helped me to develop skills on clinical research. Thank you Doan, it would not have been possible to complete this journey without the help of you.

I am extremely grateful to Prof. John Horowitz, whom kindly facilitated me with resources and his valuable advices to carry-out this clinical research. I am thankful for his support and correct direction given to me during my work at The Queen Elizabeth Hospital (TQEH) and Basil Hetzel Institute (BHI).

I would also like to give a heartfelt, special thanks to Dr. Simon Gunn, The manager, Therapeutic Research Centre, University of South Australia for his patience and valuable support given to me. He has been always there whenever I need help or advice.

I would like to thank Dr. Alicia Chan and Ms. Saifei Liu for giving some blood samples of their healthy volunteers to assess thrombospondin-1 (TSP-1) levels and use the data in Chapter 4, Section 4.4.1.1 (Cohort 1). I am thankful to SA Pathology (IMVS) for analyzing vitamin D_3 levels ($25(OH)D_3$), PTH and plasma electrolyte content of the blood samples. Special thanks goes to Mr. Andrew Fudge (Metabolic Biochemistry, Chemical Pathology Directorate, SA Pathology) for providing details on vitamin D analyzing methods. Next, I would like to thank Ms. Irene Stafford and Ms. Tamila Heresztyn, the research scientists in cardiology research laboratory, BHI for their invaluable support and technical assistance given to me in every aspect of the laboratory work. You two were big strength to me when I

was passing some hard times during my candidature. Thank you for being there for me. Furthermore, I would like to thank cardiology research nurses, Ms. Marilyn Black, Ms. Greer Dymmott, Ms. Else Jansen for helping me for paper work, recruitment and blood collection of healthy working adults whose data was used to Chapter 3 and Chapter 4 (Section 4.4.1.2, Cohort 2). I would also like to thank Dr. Tom Robertson for his support given to me during the early stages of my candidature. These acknowledgements would not be complete if I did not mention the moral support given to me by my friends in therapeutic research group and cardiology research group, I believe we made many precious memories along the way. Similarly, my thanks goes to all at department of cardiology, TQEH and BHI for the support offered to me in various ways during my stay at BHI. I would like to thank all the staff at cardiology out-patient clinics for the assistance given to me during the patient recruitment.

I would like to acknowledge the financial support provided by the University of South Australia, through the scholarship of the University of South Australia “Postgraduate Research Award”.

Of course no acknowledgement would be complete without giving heartfelt thanks to my parents, Dr. Ranjit Amarasekera and Mrs. Seetha Amarasekera. Both have instilled many admirable qualities in me and given me a good foundation with which to meet life. They have thought me about hard work and self-respect, and how to be independent. I am grateful for them both and for the "smart-genes' they passed to me. Furthermore, a big thanks to my siblings, Lesakshi and Hasitha for all the moral support given to me. I would like to acknowledge the support given by aunty Luky (Dr. Lakshmi Kumarathilaka) and uncle (Dr. Jaliya Kumarathilake) for taking care of my son during the day time of most weekdays during my candidature.

Above all, I would like to thank my husband, Rashira and my son, Minidu who was born during my candidature. They gave-up many things including my time and attention while I focused on this research project. Rashira has always been a moral supporter for me whenever I was down and being with me throughout the degree period. My son Minidu, has been a twinkling gem (as his name implies) in my eyes since he was born. I really appreciate the way they both handle this.

Last, but certainly not least, I must acknowledge various help and moral support given to me by my friends who were always been there for me whenever I need them. Thank you.

Publications arising from this work

Work presented in Chapter 2 and Chapter 4 has been published in the form of abstracts in following journals.

Amarasekera AT, Black M, Jansen E, et al, Abstract 12335: Vitamin D supplementation in healthy adults lowers plasma thrombospondin-1 levels: A novel vitamin D target?. *Circulation.* 2013; 128:A12335.

Amarasekera AT, Sverdlov AL, Roberts MS, et al, Elevated parathyroid hormone predicts high asymmetric dimethylarginine (ADMA) concentrations; independent of vitamin D status. *Eur Heart J*. 2013; 34 (suppl 1):613.

Conference presentations

Amarasekera AT, Sverdlov AL, Roberts MR, Horowitz JD, Ngo DT. 'Parathyroid hormone levels predict increased asymmetric dimethylarginine concentrations in obese diabetic patients', poster presentation at 6th international ADMA symposium 2012, Imperial College London.

Amarasekera AT, Sverdlov AL, Roberts MS, Horowitz JD, Ngo DT. 'Elevated parathyroid hormone predicts high asymmetric dimethylarginine (ADMA) concentrations; independent of vitamin D status', poster presentation at European Society of Cardiology (ESC) Congress, 2013, Netherlands.

Amarasekera AT, Black M, Jansen E, Dymmott G, Roberts MS, Chirkov YY, Horowitz JD, Ngo DT. 'Vitamin D supplementation in healthy working adults lowers plasma thrombospondin-1 levels: a novel vitamin D target?', poster presentation at American Heart Association (AHA) Scientific Sessions 2013, Dallas, Texas, USA.

Preface

This thesis is submitted as a traditional chapter format but some conference published research is also incorporated into the thesis, which is in accordance with the University of South Australia's academic regulations for Higher Degrees by Research clause 16.1.1.b. This consists of a combination of work that has been presented in international cardiovascular conferences and abstracts published in the corresponding journals (Chapter 2 and Chapter 4) and conventional chapters. All of the work including interviewing the study subjects (patients and healthy volunteers) and their recruitment, measurement of biological markers including asymmetric dimethylarginine (ADMA) and thrombospondin 1 (TSP-1), data collection, data entry, database maintenance, statistical data analysis and writing of the thesis was performed by myself, as is evidenced by first authorship of the abstracts published and the conference presentations. Assistance from my supervisors is recognized in the co-authorship of the abstracts and the presentations.

The focus of this thesis is on assessing the effects of vitamin D deficiency on endothelial function and the correction of vitamin D deficiency on markers of endothelial dysfunction. Previously, our group (Vascular Disease and Therapeutics Research Group, Basil Hetzel Institute, The Queen Elizabeth Hospital) have demonstrated that low 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentrations are associated with increased concentrations of asymmetric dimethylarginine (ADMA), an endothelial nitric oxide synthase (eNOS) enzyme inhibitor and biomarker of increased cardiovascular (CV) morbidity and mortality in healthy ageing individuals. However, it was not determined whether this relationship was independent of parathyroid hormone (PTH) levels. Three main aspects of this relationship between low vitamin D levels and endothelial dysfunction were assessed.

The first aspect was addressed in evaluating whether this newly establish relationship between vitamin D levels and ADMA concentrations is present in a high risk cohort of obese diabetics (characterized by high cardiovascular incidences, high levels of ADMA and low vitamin D levels) and whether this relationship is independent of PTH levels. This was addressed in n=164 obese diabetic patients who were recruited from out-patient clinics of TQEH and the study is described in Chapter 2. This work has been published as an abstract: "Amarasekera AT, Sverdlov AL, Roberts MS, et al, *Elevated parathyroid hormone predicts high asymmetric dimethylarginine (ADMA) concentrations; independent of vitamin D status.*

Eur Heart J. 2013; 34 (suppl 1):613" with modifications including data from ageing population added (the data was collected by Dr. Doan Ngo).

The beneficial effects of vitamin D supplementation on vascular endothelial function have also been demonstrated by various studies. Currently, the outcomes of vitamin D supplementation on plasma ADMA concentrations have not been evaluated. Thus, a second aspect was addressed in assessing whether, 1) vitamin D supplementation decreases ADMA concentrations, and 2) changes in PTH levels correlated with changes in ADMA concentrations, in vitamin D insufficient but otherwise healthy individuals free from CV risk factors. Chapter 3 addressed this in n=35 healthy working adults recruited from TQEH staff who were vitamin D insufficient and subsequently received vitamin D_3 supplementation for 12 weeks.

The third aspect assessed the relationship between vitamin D and plasma TSP-1 concentrations (an another potent inhibitor of NO-cGMP signaling pathway) in healthy volunteers and the effects of vitamin D_3 supplementation on plasma TSP-1 levels and other markers of NO signaling in subjects with low vitamin D levels (Chapter 4). To evaluate the relationship between vitamin D and plasma TSP-1 concentrations, n=58 healthy subjects were assessed (cohort 1, blood samples were collected by Dr. Alicia Chan and Ms. Saifei Liu). An assessment of the effects of vitamin D_3 supplementation on plasma TSP-1 levels in healthy subjects with low vitamin D levels was carried out by measuring baseline and follow-up TSP-1 levels of the subjects (cohort 2) recruited to the previous study in Chapter 3. At this stage the blood samples of n=31 (n=4 blood samples were heamolysed from total of 35 collected) healthy subjects with low vitamin D levels were assessed for their TSP-1 levels at the baseline and follow-up visits after the vitamin D_3 supplementation. This work has been published as an abstract: "Amarasekera AT, Black M, Jansen E, et al, Abstract 12335: *Vitamin D supplementation in healthy adults lowers plasma thrombospondin-1 levels: A novel vitamin D target?.* Circulation. 2013; 128:A12335" and the study is described in detail in Chapter 4.

Chapter 1: Literature review and thesis objectives

Chapter 2: Evaluating the prevalence of vitamin D deficiency and relationship between low vitamin D and elevated parathyroid hormone on markers of endothelial function and inflammation in obese-diabetic patients

Chapter 3: Effects of vitamin D_3 supplementation and change of PTH status on plasma concentrations of asymmetric dimethylarginine in healthy working adults

Chapter 4: Vitamin D supplementation in healthy adults lowers plasma thrombospondin-1 levels: A novel vitamin D target?

Chapter 5: Summary, conclusions and future perspectives

Chapter 1 Literature review and thesis objectives

1.1. Synopsis

Vitamin D deficiency is a rising worldwide pandemic and has been associated with various health consequences. The vitamin D receptor (VDR) is widely expressed with a broad spectrum of physiological activities of vitamin D. A growing body of evidence suggests that vitamin D is important in cardiovascular homeostasis and conversely, that vitamin D deficiency may represent a risk factor for the development of coronary diseases. Low serum vitamin D levels are associated with elevated parathyroid hormone levels and vitamin D deficiency and secondary hyperparathyroidism, either in combination or independently, have been associated with increased cardiovascular (CV) events. However, mechanistic insights of these findings remain largely elusive. Elevated oxidative stress, inflammatory activation, impaired endothelial function and insulin resistance have been described by previous studies as possible mechanisms for the adverse cardiovascular outcomes in low vitamin D and elevated parathyroid hormone status. Our group has previously demonstrated that low 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentrations are associated with increased concentrations of asymmetric dimethylarginine (ADMA), an eNOS inhibitor and biomarker of increased CV morbidity and mortality in healthy ageing individuals. This thesis will examine the following: 1) the relationship between ADMA and $25(OH)D_3$ in patients with diabetes and obesity, in relation with PTH status, 2) determine whether vitamin D supplementation decreases ADMA levels in vitamin D deficient but otherwise healthy individuals, and 3) the effects of vitamin D deficiency and its restoration on another inhibitor of nitric oxide signaling, TSP-1.

1.2 Vitamin D_3 – Introduction

Vitamin D is a fat soluble prohomone: the two forms of which are vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol). The main sources of vitamin D are sunlight, diet and vitamin D supplements (Lee, J.H. et al. 2008).

1.2.1 Biosynthesis and metabolism of vitamin D_3

Vitamin D_2 (ergocalciferol) is obtained from irradiation of the yeast or plant sterol ergosterol. Vitamin D_3 (cholecalciferol) is produced in human skin or can be found in oily fish. Solar ultraviolet B (UVB) radiation penetrates the skin and photolytically converts 7-dehydrocholesterol (provitamin D_3) to previtamin D_3, which is rapidly converted to vitamin D_3. Furthermore, UV exposure leads to photo-isomerisation of previtamin D_3 to biologically inert isomers; tachysterol, a relatively unstable irradiation product, and lumisterol, thus excessive exposure to sunlight does not cause vitamin D_3 intoxication (Holick, M.F. 2007; Lee, J.H. et al. 2008).

Vitamin D_3 is formed in the skin and transported in the circulation by binding to the plasma transport protein, vitamin D binding protein (DBP). The DBP is the major plasma carrier protein of vitamin D and its metabolites, 25(OH)D_3 (calcidiol) and 1,25$(OH)_2D_3$ (calcitriol) (Cooke, NE & Haddad 1989; White & Cooke 2000). The functions of DBP are binding, solubilization and serum transport of vitamin D and its steroidal metabolites (Cooke, NE & Haddad 1989; Speeckaert et al. 2006).

Vitamin D_3 from the skin and diet is metabolized in the liver by the enzyme 25-hydroxylase (CYP27A1) to 25-hydroxyvitamin D_3 (25(OH)D_3), which is the major circulating metabolite of vitamin D and is a marker of vitamin D status (Lee, J.H. et al. 2008; Miller, WL & Portale 2000; Prosser & Jones 2004). Then, 25-hydroxyvitamin D_3 is transported to the kidneys and is converted by 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) enzyme to 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the active metabolite of vitamin D_3 (Lee, J.H. et al. 2008; Miller, WL & Portale 2000). The renal production of 1,25-dihydroxyvitamin D_3 is tightly regulated by plasma parathyroid hormone levels and serum calcium and phosphorus levels (Lee, J.H. et al. 2008). PTH enhances the absorption of calcium from the intestine by stimulating the 1-α-hydroxylase enzyme to produce 1,25$(OH)_2D_3$, the active form of vitamin D_3, which in turns negatively inhibits further production of PTH in a feedback mechanism. Within the promoter of the PTH gene, there is a region that binds to the vitamin D receptor (VDR) and mediates the suppression of the PTH promoter by 1,25$(OH)_2D_3$ (Silverberg & Bilezikian 1999). A low parathyroid hormone level and high serum calcium and phosphorus levels induce the renal expression of the enzyme 25-hydroxyvitamin D-24 hydroxylase (CYP24A1), which catabolizes both 25(OH)D_3 and 1,25$(OH)_2D_3$ to biologically inactive, water-soluble calcitroic acid which is reabsorbed by the liver followed by biliary excretion

(Andersson et al. 1989; Prosser & Jones 2004). Figure 1.1 summarises the biosynthesis and metabolism of vitamin D_3.

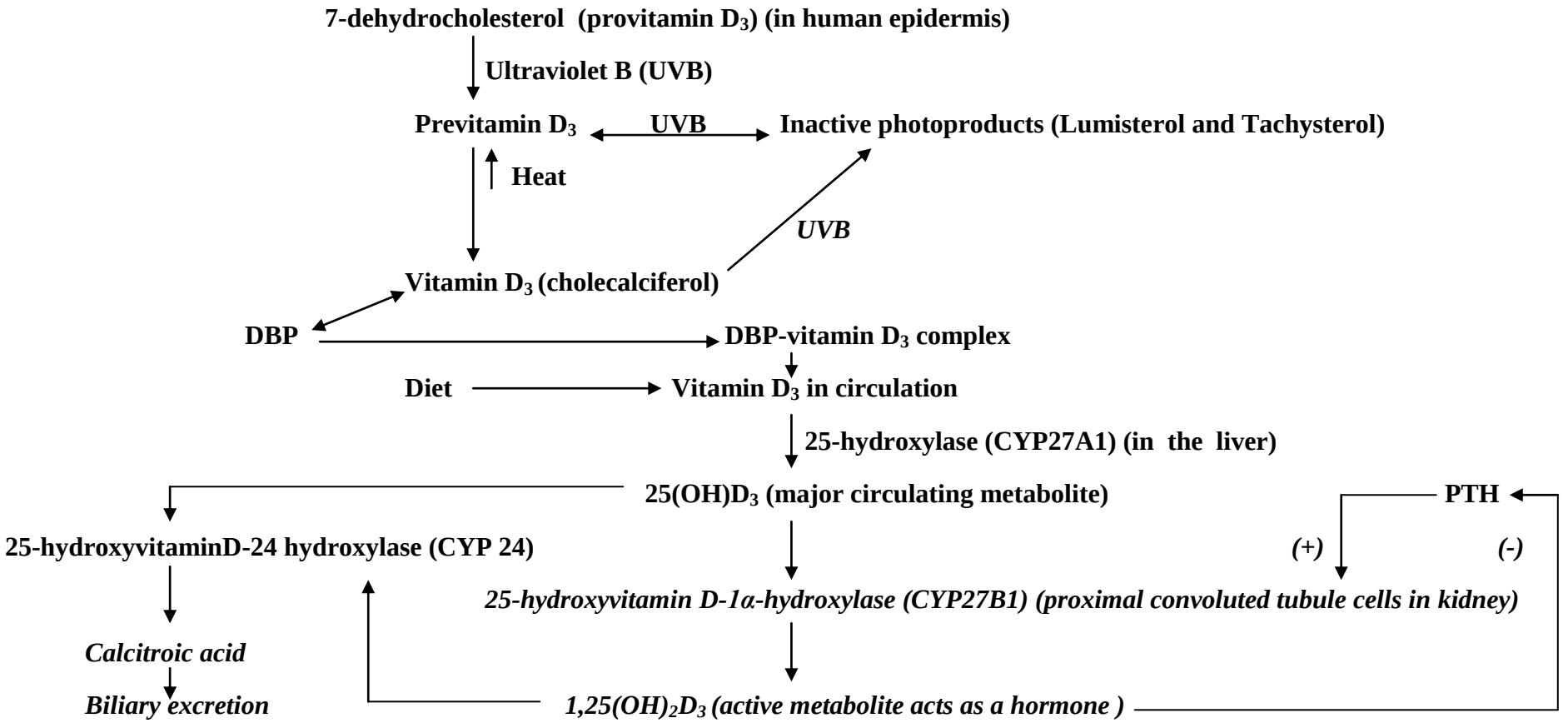

Figure 1.1: Biosynthesis and metabolism of vitamin D_3 (Andersson et al. 1989; Baudino et al. 1998; Bourguet et al. 1995; Cooke, NE & Haddad 1989; Haussler et al. 1995; Miller, WL & Portale 2000; Prosser & Jones 2004; Reddy et al. 2007; Silverberg & Bilezikian 1999; Speeckaert et al. 2006; White & Cooke 2000; Zehnder et al. 2002).

1.2.2 Circulating 25(OH)D_3 levels – a marker of vitamin D status

Clinical studies measure circulating 25(OH)D_3 levels as a marker of vitamin D status, even though 1,25$(OH)_2D_3$ is the biologically active form of vitamin D. There are several reasons to use 25(OH)D_3 levels as a marker of vitamin D status;

a 25(OH)D_3 is the major circulating metabolite which has a half-life of 2-3 weeks, whereas 1,25$(OH)_2D_3$ has a half-life of only 4-6 hours.

b The active metabolite of vitamin D, 1,25$(OH)_2D_3$ circulates at 1/1000 fold lower concentration than 25(OH)D_3.

c 25(OH)D_3 is bio-synthesized from both vitamin D intake and vitamin D that is produced from sun exposure.

d The lower ionized calcium levels in vitamin D deficiency increases the production and secretion of PTH, which in turn increases the renal production of 1,25$(OH)_2D_3$. Thus, as the subject becomes vitamin D deficient, the increased PTH levels result in normal or elevated levels of 1,25$(OH)_2D_3$. This makes the 1,25$(OH)_2D_3$ level unsuitable as a marker of vitamin D status (Holick, M.F. 2009).

Therefore, serum concentrations of the circulating metabolite of vitamin D_3, 25(OH)D_3, are considered as the best indicator of vitamin D status (Holick, M.F. 2009).

Although commonly used for the measurement of an individual's serum vitamin D status, the reference range for vitamin D is currently established for skeletal health, not for other non-skeletal conditions. The circulating 25(OH)D_3 levels found to suppress the maximal serum PTH concentration has been proposed and used by several studies (Holick, Michael F 2004; Holick, Michael F. et al. 2012; Simonelli et al. 2005). The vitamin D levels required for PTH activation for bone health are also dependent on a number of factors including age, gender, genetics, and renal function (Holick, Michael F. et al. 2012). The concentrations of 25(OH)D_3 required for PTH levels to plateau range from 20ng/mL to 39ng/mL (50-97nmol/L) depending on gender and age, with men generally requiring higher serum 25(OH)D_3 concentrations than women (Holick, Michael F. et al. 2012). Current literature available on cut-off points of serum 25(OH)D_3 concentrations for vitamin D deficiency/insufficiency in relation to various health outcomes are summarized in Table 1.1. Thus, there is currently no

strict agreement on the reference value of serum $25(OH)D_3$ levels. Most experts define vitamin D insufficiency as a $25(OH)D_3$ level between 21 to 29 ng/ml (50-74 nmol/L) and vitamin D deficiency as a $25(OH)D_3$ level < 20 ng/ml (50 nmol/l). The optimal level of $25(OH)D_3$ is considered to be 30 ng/ml (75 nmol/L) or higher (Lee, J.H. et al. 2008). Therefore, the criteria used in this thesis for vitamin D deficiency was $25(OH)D_3 < 50$ nmol/L and vitamin D insufficiency was $25(OH)D_3 < 75$ nmol/L.

There are a substantial number of reports concerning the significant inter-laboratory variability for vitamin D concentration measurement (Binkley et al. 2004; Glendenning et al. 2003). The differences could primarily be influenced by the different methods used for measurement and the lack of standardization between laboratories. It is also highly debatable which assay provides the "most accurate" results and whether or not it is appropriate to compare the results to the All-Laboratory Trimmed Mean (ALTM), which has been used as a consensus mean for decades, due to the large variability of assay methods used (Carter 2009). Conversely, the differences in assay calibration between different laboratories, which presents as another potential issue, could perhaps be improved by the use of a single calibrator (Binkley et al. 2008). Overall, a rigorous standardization of measurement of vitamin D is warranted in this area to facilitate proper diagnosis and treatment.

Table 1.1: Current literature available on cut-off points of serum 25(OH)D_3 concentrations for vitamin D deficiency in relation to various health outcomes

Study / Literature	Sample size	Patient type	25-hydroxyvitamin D_3 cut-off level for deficiency nmol/l / (ng/ml)
Holick et al (Holick, M.F. et al. 2005)	1,536	Postmenopausal women, osteoporosis	<75 (30)
Simonelli et al (Simonelli et al. 2005)	82	Nontraumatic fracture	<75 (30)
Aloia et al (Aloia et al. 2006)	208	Healthy women	< 44 (17.5)
Bischoff-Ferrari et al (Bischoff-Ferrari et al. 2006)	Review		< 75 (30)
Forman et al (Forman et al. 2007)	1,811	Prospective cohort studies including men & women	< 37.7 (15)
Tai et al (Tai et al. 2008)	Review		< 50 (20)
Sugden et al (Sugden et al. 2008)	87	Type 2 diabetes mellitus patients	<50 (20)
Holick et al (Holick, M.F. 2007, 2009; Holick, M.F. & Chen 2008; Lee, J.H. et al. 2008)	Review		< 50 (20)
Tarcin et al (Tarcin et al. 2009)	23	Asymptomatic healthy adults	< 25 (10)
Anderson et al (Anderson, Jeffrey L. et al. 2010)	41,497	Analysis of electronic medical records database (2000-2009)	≤ 37.7 (15)

Study / Literature	Sample size	Patient type	25-hydroxyvitamin D_3 cut-off level for deficiency nmol/l / (ng/ml)
May et al (May et al. 2010)	7,358	Patients ≥50 years of age, with a CV diagnosis and no prior depression diagnosis	< 50 (20)
Jablonski et al (Jablonski et al. 2011)	47 28	Middle-aged/older (50 to 79 years) men; postmenopausal women;	< 50 (20)
Yiu et al (Yiu, YF et al. 2011)	280	Type 2 diabetes mellitus patients recruited in outpatient clinics	< 50 (20)
Harris et al (Harris et al. 2011)	57	Healthy African- American men and women	< 50 (20)
Annweiler et al (Annweiler et al. 2012)	92	Caucasian community-dwellers with no clinical hydrocephalus	50 (20)
Mahdavi et al (Mahdavi et al. 2013)	106 110	Patients with non-ST elevation myocardial infarction and high-risk unstable angina; Patients with ST elevation myocardial infarction;	≤ 50 (20)
Ott et al (Ott et al. 2012)	31	Hypercholesterolemic patients with vitamin D insufficiency	< 75 (30) (insufficiency)

Study / Literature	Sample size	Patient type	25-hydroxyvitamin D_3 cut-off level for deficiency nmol/l / (ng/ml)
Bener et al (Bener & El Ayoubi 2012)	635	Patients with breast cancer	< 25(10) (severe) 25-50 (10–19) (moderate)
Coutard et al (Coutard et al. 2013)	226	Geriatric patients (aged ≥70 years)	<50 (20)
Bilinski et al (Bilinski & Boyages 2012)	214	Women newly diagnosed with breast cancer	<25 (10) (severely deficient) 25-49 (10-20) (deficient)
Fridman et al (Fridman et al. 2012)	60	HIV-positive patients	< 25 (10)
Guasch et al (Guasch et al. 2012)	316	Obese patients	25-75(10-30) (insufficient) <25 (10) (deficient)
Beloyartseva et al (Beloyartseva et al. 2012)	2,119	Healthy medical and paramedical personnel from India	< 50 (20)
Leedahl et al (Leedahl et al. 2013)	548	Psychiatric patients	< 25(10) (severe) 25-60 (10–24) (moderate)
Sokol et al (Sokol et al. 2012)	90	Patients with coronary artery disease	< 50 (20)
Correia et al (Correia et al. 2013)	206	Admitted for unstable angina, non–ST-segment elevation myocardial infarction, or ST-segment elevation acute myocardial infarction	≤ 25 (10)

Study / Literature	Sample size	Patient type	25-hydroxyvitamin D_3 cut-off level for deficiency nmol/l / (ng/ml)
Amrein et al (Amrein et al. 2012)	480	Mixed population of critically ill patients	≤ 50 (20)
Simba et al (Simha et al. 2012)	12	Healthy subjects	< 50 (20)
Gradinaru et al (Gradinaru et al. 2012)	30	Elderly subjects with impaired fasting glucose	< 75 (30)
Schierbeck et al (Schierbeck et al. 2012)	2,016	Healthy, recently postmenopausal women	<50 (20)
Syal et al (Syal et al. 2012)	100	Patients undergoing coronary angiography	< 25 (10)
Van de Luijtgaarden et al (van de Luijtgaarden et al. 2012)	254 236	Symptomatic peripheral arterial disease aortic aneurysm	≤ 25 (10) (severe) 26-50 (10.3-20) (moderate)
Runia et al (Runia et al. 2012)	73	Patients with relapsing-remitting multiple sclerosis	<50 (20)
McCue et al (McCue et al. 2012)	107	Psychiatric in patients aged 18 years and older	≤ 50 (20)
Yilmaz et al (Yilmaz et al. 2012)	171	Type 2 diabetes mellitus patients	< 50 (20)

Study / Literature	Sample size	Patient type	25-hydroxyvitamin D_3 cut-off level for deficiency nmol/l / (ng/ml)
Yiu et al (Yiu, Y-F et al. 2013)	100	Type 2 DM with suboptimal vitamin D levels	50 (20)
Macdonald et al (Macdonald et al. 2013)	365	Healthy postmenopausal women	< 25 (10)
McAlindon et al (McAlindon T & et al. 2013)	146	Symptomatic knee osteoarthritis	90 (36)
Carrara et al (Carrara et al. 2014)	15	Essential hypertension & hypovitaminosis	75 (30)
Turer et al (Turer, Lin & Flores 2013)		6- to 18-year-old children	< 50 (20)
McAree et al (McAree et al. 2012)	346	Unselected multi-ethnic population of pregnant women	< 25 (10)
Zittermann et al (Zittermann, A. et al. 2013)	4418	Cardiac surgical patients	< 30 (12)

1.2.3 Vitamin D deficiency

1.2.3.1 Incidence of vitamin D deficiency

Vitamin D deficiency, defined generally as a 25(OH)D_3 level < 20 ng/ml (50 nmol/l), is a rising pandemic worldwide. Recently, vitamin D deficiency has been associated with many health risks and is present in approximately 30% to 50% of the general population (Lee, J.H. et al. 2008). Holick et al reported the presence of vitamin D deficiency in adults is up to 50% (Tangpricha et al. 2002) while the Third National Health and Nutrition Examination Survey (NHANES) data suggested the occurrence of vitamin D deficiency in the United States to be 25% to 57% of adults (Looker et al. 2002).

Several studies have assessed the prevalence of vitamin D deficiency in Australia (Agarwal et al. 2011; Barnett, De Looper & Fraser 2008; Nowson & Margerison 2002). The highest rates of deficiency occur in dark-skinned or veiled pregnant women (80%) (Grover & Morley 2001), mothers of infants treated for rickets (Nozza & Rodda 2001), and elderly residents in residential care facilities (97%) (Flicker et al. 2003). Nowson and Margerison reported the prevalence of vitamin D deficiency as 23% in young Australian adults (Nowson & Margerison 2002).

1.2.3.2 Risk factors for vitamin D deficiency

Vitamin D levels are affected by a number of factors including the time of the day, season, latitude, age and skin pigmentation. The amount of 7-dehydrocholesterol in the epidermis declines in later life and hence the elderly produce less vitamin D_3 than young adults. On the other hand, increased skin melanin pigmentation increases the amount of UVB photon absorption and hence longer exposure to sunlight is required to make the same amount of vitamin D_3 compared to lighter-skinned people (Holick, M.F. 2004).

Vitamin D deficiency has become a more prevalent condition because of the use of sunscreens and indoor lifestyles. One study demonstrated that an increase of serum vitamin D_3 levels was higher in unprotected subjects than that of subjects protected by sunscreen. Matsuoka et al. demonstrated that para-aminobenzoic acid in sunscreen prevented the photoisomerization of 7-dehydrocholesterol to previtamin D_3 in human skin slices in vitro

(Matsuoka, LY et al. 1987). Furthermore, increased skin pigment can greatly reduce the UV radiation-mediated synthesis of vitamin D_3. Thus, ethnic groups with darker skin synthesize lower amounts of vitamin D when compared to people with lighter skin who are exposed to an equivalent standard dose of ultraviolet radiation (UVR). An early study showed that women with darker skin produce a substantially lower quantity of $25(OH)D_3$ than that of white women at all times of the year, although both groups show seasonal variations in $25(OH)D_3$ levels (Clemens et al. 1982).

Another factor that could affect vitamin D status is body fat content. It has been suggested that obese patients (BMI $\geq$ 30 Kg/m^2) are more likely to require a larger than usual dose of vitamin D_3 (Gallagher, Yalamanchili & Smith 2013). Vitamin D is a fat soluble vitamin and is readily sequestered into fat stores (Blum et al. 2008). It has been found that when given the same dose of vitamin D (50,000 IU) obese patients had a significantly smaller increase in plasma vitamin D concentration compared to non-obese patients (Wortsman et al. 2000). Furthermore, the prevalence of vitamin D deficiency ($25(OH)D_3$ level < 50 nmol/L) in morbidly obese patients was reported to be as high as 61% and 90% for combined vitamin D deficiency and insufficiency ($25(OH)D_3$ level < 75 nmol/L) (Goldner et al. 2008).

Patients with Type 2 diabetes mellitus have been found to have an increased prevalence of vitamin D deficiency (Muscogiuri et al. 2012). More recently, a number of observational studies reported the possible association between lower vitamin D status and higher risk of type 2 diabetes (Knekt et al. 2008; Mitri, Muraru & Pittas 2011; Pittas, A.G. et al. 2007). A study carried out with elderly non-insulin dependent diabetes mellitus (NIDDM) participants demonstrated that 39% were vitamin D depleted (Baynes et al. 1997). A large prospective study carried out with 83779 women who were free from diabetes and cardiovascular diseases at the baseline reported that a combined intake of calcium and vitamin D was associated with a 33% lower risk of type 2 diabetes (Pittas et al. 2006). Another cohort study consisted of 4423 of men and women aged 40-69 years and reported a higher risk of type 2 diabetes with individuals who were low in their vitamin D levels at baseline (Mattila et al. 2007).

1.2.4 Vitamin D_3 – mechanisms of physiological action

Vitamin D can exert physiological actions via genomic effects through nuclear vitamin D_3 receptors or non-genomic effects which are mediated via membrane bound vitamin D receptors.

1.2.4.1 Genomic effects of vitamin D_3

The genomic effects of vitamin D_3 are exerted largely through the nuclear vitamin D_3 receptors (VDR). The active metabolite of vitamin D, $1,25(OH)_2D_3$, acts as a hormone and performs its biological actions by complexing with VDR (Reddy et al. 2007). These nuclear VDRs are distributed widely in a wide range of tissue types (Baudino et al. 1998; Reddy et al. 2007). The VDR is a ligand-dependent transcription activator that modulates the expression of vitamin D-sensitive genes. Interaction between the active form of vitamin D and VDR results in conformational changes of the receptor, which allow the VDR to form a heterodimer with retinoid X receptor (RXR) (Bourguet et al. 1995). The heterodimer complex binds to vitamin D-responsive elements (VDREs), which is a DNA sequence that is found in the promoter region of vitamin D regulated genes. This exposes new hydrophobic surfaces needed to interact with other nuclear factors and attracts a complex of proteins called co-activators of the VDR-RXR complex. The co-activator complexes cover the gap between VDRE and RNA polymerase II and help with transcription. Gene transcription is initiated to produce the corresponding mRNA, which leaves the nucleus to translate the corresponding protein (Haussler et al. 1995).

1.2.4.2 Non-genomic effects of vitamin D_3

The non-genomic effects of $1,25(OH)_2D_3$ are more rapid and may be mediated by membrane bound vitamin D receptors which are not involved with gene regulation (Lips, P 2006; Norman, Anthony W et al. 1992). $1,25(OH)_2D_3$ has been reported to have non-genomic effects such as regulation of calcium and chloride channel activity, protein kinase C activation and distribution, and phospholipase C activity in a number of cells including osteoblasts (Baran, Daniel T et al. 1991), liver (Baran, Daniel T. et al. 1990), muscle (Morelli, De Boland & Boland 1993), pancreatic β-cells (Kajikawa et al. 1999), vascular

smooth muscle cells (Rebsamen et al. 2002), monocytes (Berry 1999) and intestine (Khare et al. 1997; Wali et al. 1990). Figure 1.2 summarises the mechanisms of action of vitamin D_3.

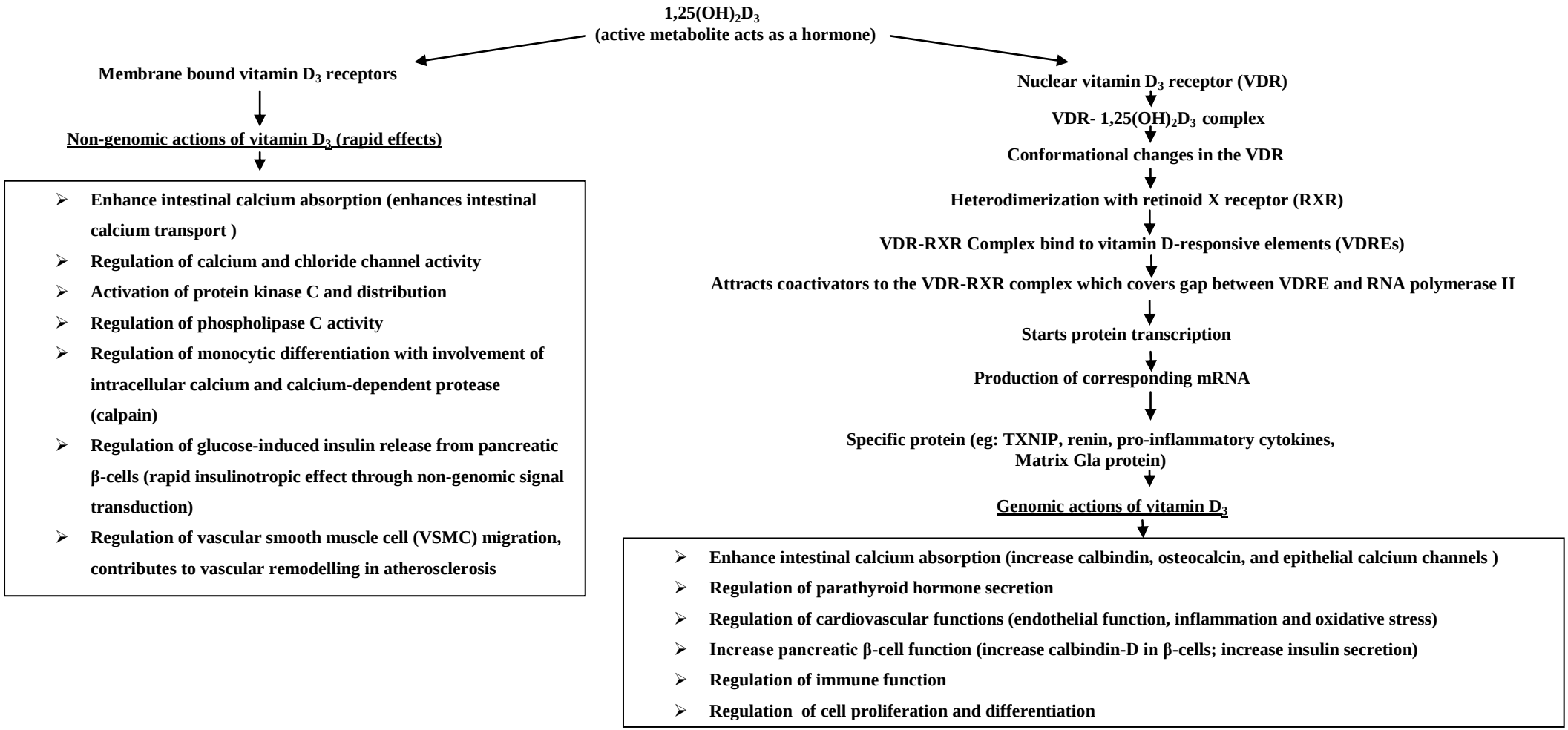

Figure 1.2: Mechanisms for effects of vitamin D_3 (Andersson et al. 1989; Baudino et al. 1998; Bourguet et al. 1995; Cooke, NE & Haddad 1989; Haussler et al. 1995; Miller, WL & Portale 2000; Prosser & Jones 2004; Reddy et al. 2007; Silverberg & Bilezikian 1999; Speeckaert et al. 2006; White & Cooke 2000; Zehnder et al. 2002).

1.2.5 Physiological effects of vitamin D_3

Vitamin D receptors have widespread tissue distribution. The wide tissue distribution of VDR is associated with wide spectrum of activities including newly identified roles of vitamin D.

1.2.5.1 Classical effects of vitamin D_3

The well-recognised classical effects of vitamin D include the regulation of calcium and phosphate absorption as well as the regulation of parathyroid hormone (PTH) secretion.

1.2.5.1.1 Regulation of calcium and phosphate homeostasis

One of the oldest and best known actions of vitamin D is to promote intestinal calcium absorption, first described in vitro by Schachter and Rosen in 1959 and in vivo by Wasserman et al. in 1962 (Schachter & Rosen 1959; Wasserman, RH 1962).

Research showed that the active vitamin D metabolite, $1,25(OH)_2D_3$ and VDR are essential for intestinal calcium absorption (Bouillon, Van Cromphaut & Carmeliet 2003; Heaney et al. 2003; Lips, P 2006; Van Cromphaut et al. 2001). Absorption of calcium from the luminal contents of the intestine involves both transcellular and paracellular pathways (Bouillon, Van Cromphaut & Carmeliet 2003). The transcellular pathway dominates in the duodenum, and this is the pathway primarily regulated by a combination of genomic and nongenomic actions of $1,25(OH)_2D_3$ (Bouillon, Van Cromphaut & Carmeliet 2003; Lips, P 2006).

The genomic mechanisms included the up-regulation of gene expression of vitamin D-dependent calcium binding protein (calbindin) and osteocalcin (Christakos, Gabrielides & Rhoten 1989; Lian et al. 1989; McDonnell et al. 1989; Wasserman, R & Taylor 1966), which in turns increases the intestinal calcium absorption (Friedlander, Henry & Norman 1977). Calcium binding protein facilitates the diffusion of calcium through the cytosol and serves as an intracellular calcium buffer to keep the cytoplasmic calcium concentrations at the optimal levels. Epithelial calcium channels are very sensitive to endogenous $1,25(OH)_2D_3$. Expression of epithelial calcium channels is markedly down-regulated in VDR knock-out mice and shown to increase the expression with exogenous administration of $1,25(OH)_2D_3$ (Bouillon, Van Cromphaut & Carmeliet 2003).

The non-genomic mechanism of calcium absorption involves plasma membrane VDRs and second messengers such as cyclic AMP. At the basolateral membrane, the calcium is pumped out of the cells by ATP-dependent Ca^{2+} pump through CaATPase, an enzyme induced by $1,25(OH)_2D_3$ in the intestine. $1,25(OH)_2D_3$ enhances intestinal calcium transport by inducing TRPV6 (previously known as CaT1) calcium channel, calbindin (CaBP), and Ca-ATPase as well as increasing the amount of CaM bound to BBMI in the brush border.

Intestinal phosphate transport is also under the control of vitamin D. Active phosphate transport is greatest in the jejunum and phosphate transport at both the brush border and basolateral membranes requires sodium. The expression of sodium-phosphate transporters in the small intestine (NaPi-IIb) is increased by $1,25(OH)_2D_3$ (Rizzoli, Fleisch & Bonjour 1977).

1.2.5.1.2 Regulation of parathyroid hormone secretion

Parathyroid hormone (PTH) is the most important endocrine regulator of calcium and phosphorus concentrations in the extracellular fluid. Calcium is the principal regulator of parathyroid glandular activity. The secretion rate of PTH is inversely associated with extracellular calcium ion concentration. Parathyroid hormone is released from cells of the parathyroid glands in response to low extracellular concentrations of free calcium and bring the calcium levels back to normal by the mobilization of calcium from bone, enhancing the absorption of calcium from the small intestine, and suppression of calcium loss in the urine (Fleet et al. 1994; Rasmussen 1961).

Increased levels of $1,25(OH)_2D_3$ inhibit the synthesis and secretion of PTH (Demay et al. 1992). Within the promoter of the PTH gene, there is a region that binds with the VDR and mediates the suppression of the PTH promoter by $1,25(OH)_2D_3$ (Demay et al. 1992). Elevated levels of $1,25(OH)_2D_3$ enhance the calcium sensing receptor of PTH which sensitizes the parathyroid gland to calcium and therefore inhibits further PTH synthesis and reduces calcium absorption (Canaff & Hendy 2002). As PTH promotes the production of $1,25(OH)_2D_3$ by stimulating the 1 α hydroxylase enzyme, this inhibition of PTH synthesis and secretion provides an important negative feedback loop. These actions of PTH are used clinically to treat the consequences of secondary hyperparathyroidism in renal failure.

1.2.5.2 Non-classical effects of vitamin D_3

The VDR are widely expressed and this is associated with a broad spectrum of non-classical effects of vitamin D. Literature on the possible non-classical effects of vitamin D including its possible protective effects against cancer (Garland & Garland 1980), the cardiovascular system (Scragg, Robert 1981) and pancreatic insulin secretion (Norman, A.W. et al. 1980) have all been reported in early studies. Furthermore, there have been numerous studies which have showed the association of vitamin D deficiency and various health complications (Bolland, M.J. et al. 2010; Chiu, K.C. et al. 2004; Holick, M.F. 2004; Holick, M.F. & Chen 2008; Lips, P. 2001; Mathieu, C. et al. 2005; Norman, A.W. et al. 1980; Pittas et al. 2006; Zella & DeLuca 2003).

1.2.5.2.1 Effects of vitamin D_3 on pancreatic β-cell function

It has been suggested that vitamin D has effects on insulin secretion. An early study carried out by Norman et al. (Norman, A.W. et al. 1980) showed for the first time a possible link between vitamin D status and pancreatic insulin secretion. This study showed that during a 30 min period of perfusion with glucose and arginine there was a 48% reduction in insulin secretion in vitamin D-deficient rats, which was reversible with vitamin D supplementation.

However, the effects of reduced vitamin D status and the action of vitamin D on pancreatic β cell function have been conflicting. Vitamin D deficiency has been shown to impair insulin synthesis and secretion in humans and animal models. Data from VDR knock-out mice have shown inconsistent results with some groups reporting an improvement in glucose tolerance (ZEITZ et al. 2003) while others have reported no improvement in glucose metabolism (Mathieu, Chantal et al. 2001).

Nonetheless, previous studies carried out with experimental animal models demonstrated promising effects of vitamin D supplementation on pancreatic β cell function. An experiment which was conducted with vitamin D deficient male rats, demonstrated that the administration of the active metabolite of vitamin D_3, $1,25(OH)_2D_3$ stimulates insulin secretion when compared to the vitamin D deficient control group or the deficient group of rats which received vitamin D_3 or another vitamin D_3 metabolite, 24R, $25(OH)_2D_3$ irrespective of the dietary caloric intake and prevailing serum calcium levels (Kadowaki & Norman 1985). Furthermore, $1,25(OH)_2D_3$ has been shown to increase insulin secretion by

promoting a significant rise in cytosolic Ca^{2+} levels in islet cells (Mathieu, C. et al. 2005). Studies conducted with isolated pancreatic islet cells showed that insulin synthesis and release were increased in $1,25(OH)_2D_3$-treated cultures (d'Emden et al. 1989). In this study, neonatal islets which were treated with $1,25(OH)_2D_3$ (10^{-8} mol/L) showed a 2.5-fold increase in insulin levels ($p<0.001$). These data showed the direct effect of $1,25(OH)_2D_3$ on β cell function. In addition, VDR and vitamin D-induced calcium binding protein (calbindin-D) have been found in pancreatic β cells and studies have demonstrated that calbindin-D by regulating intracellular calcium can depolarize the β cell membrane, and induce insulin secretion (Sooy et al. 1999). Calbindin-D acts as a buffer of calcium, which prevents the activation of cytokine and cytokine-mediated β cells destruction (Rabinovitch et al. 2001). Another mechanism suggested for the stimulatory effects of $1,25(OH)_2D_3$ on islet cell insulin synthesis may involve increased transcriptional activity of the insulin gene or increased insulin mRNA stability (ZEITZ et al. 2003). It was also demonstrated that in mice with mutant dysfunctional VDR there was a significant impairment in glucose tolerance and insulin secretion relative to wild type controls. More importantly, there was a significant decrease in insulin mRNA levels in the mutant VDR mice when compared to wild type controls independent of body weight and mineral levels (ZEITZ et al. 2003).

Furthermore, recent studies have showed promising effects of vitamin D_3 on pancreatic β-cell function. A study carried out with young non-obese vitamin D-deficient mice demonstrated that Type 1 diabetes is increased in the first months of life of non-obese vitamin D deficient mice when compared to non-obese control group. At 100 days of age, vitamin D-deficient mice were more glucose intolerant and, at 250 days, the vitamin D-deficient mice were more diabetic compared to the controls. This research found high pro-inflammatory cytokine expression in the islets of vitamin D-deficient non-obese diabetic (NOD) mice compared to control NOD mice (Giulietti et al. 2004).

Clinically, numerous observational and interventional studies have demonstrated that low serum $25(OH)D_3$ levels are inversely associated with elevated fasting glucose and insulin levels (Chiu, Ken C et al. 2004; Forouhi et al. 2008). Chiu et al (Chiu, K.C. et al. 2004) showed that healthy glucose tolerant subjects had a negative interaction of $25(OH)D_3$ concentration with post-challenge (60, 90 and 120 min) plasma glucose concentrations. On multivariate analysis, these correlations remained significant after adjustment for age, sex, ethnicity, BMI, waist-to-hip ratio, blood pressure and season. There was no correlation between $25(OH)D_3$ levels and fasting plasma glucose concentration or 30 min post-challenge

plasma glucose concentration in this cohort of healthy subjects. Another study carried out in non-diabetic subjects showed an inverse association between baseline serum 25(OH)D_3 levels and development of diabetes and insulin resistance. The baseline 25(OH)D_3 was inversely associated with 10-year risk of hyperglycemia, insulin resistance and metabolic syndrome in this cohort (Forouhi et al. 2008). A study carried out in patients with chronic kidney disease (CKD) reported that serum 25(OH)D_3 was inversely correlated with insulin resistance measured by homeostatic model assessment for insulin resistance (HOMA-IR). This association was independent of kidney function in the CKD patients (Chonchol & Scragg 2006). In addition, a study carried out with 27 polycystic ovary syndrome women and 20 age-matched controls reported that plasma 25(OH)D_3 levels directly correlated with the quantitative insulin-sensitivity check index (QUICKI), which is another marker of insulin resistance (IR), in the entire subject group. This association remained significant after multivariate analysis independent of hs-CRP, BMI, ADMA and presence of polycystic ovarian syndrome (Ngo et al. 2011).

However, data on the effects of vitamin D supplementation on insulin resistance and glucose tolerance are inconclusive. In South Asian women, vitamin D_3 supplementation of 4000 IU/daily for 6 months significantly improved insulin sensitivity and insulin resistance. In this cohort, fasting insulin levels were reduced significantly after the treatment (von Hurst, Stonehouse & Coad 2010). Furthermore, in Caucasian adults without diabetes, after 3 years of vitamin D_3 supplementation (700 IU) there was a significant lowering of fasting glucose and HOMA-IR levels (Pittas, Anastassios G et al. 2007). Conversely, some studies reported no effects of vitamin D supplements on glycaemic control. In the Nurses' Health Study, women who were free from diabetes, cardiovascular diseases or cancer at baseline, reported no association between total vitamin D intake and occurrence of Type 2 diabetes after 2-4 years of follow-up. However, the relative risk of Type 2 diabetes was higher in the lowest category of vitamin D intake (≤100 IU/day) from supplements when compared to the highest category (vitamin D intake ≥400 IU/day) also from supplements (Pittas et al. 2006). Another double-blinded, placebo-controlled, cross-over trial of vitamin D (1,25(OH)$_2D_3$ 1 µg/day) treatment carried out with diabetic subjects showed no effect on fasting or stimulated glucose or insulin levels. However, this study was conducted on only 20 subjects and the vitamin D treatment period was limited to 4 days (Orwoll, Riddle & Prince 1994).

1.2.5.2.2 Effects of vitamin D_3 on inflammation

The active form of vitamin D_3, $1,25(OH)_2D_3$ has been shown to play an important role in both innate and adaptive immunity. The VDR has been described to be present in immune cells such as human lymphocytes and monocyte-macrophage cells which have long been known to express the VDR, suggesting that vitamin D can activate immune responses (Provvedini et al. 1983; Tsoukas, Provvedini & Manolagas 1984).

Low vitamin D levels have been demonstrated to be associated with increased plasma concentrations of hs-CRP, a marker of inflammatory status and a risk factor for cardiovascular diseases (Murr et al. 2012; Ngo, D.T. et al. 2010). In agreement with previous studies (Cushman et al. 2005; Dobnig, H. et al. 2008b; Targher et al. 2006), Ngo et al demonstrated that in randomly aging subjects, high hs-CRP was associated with low $25(OH)D_3$ levels, independent of age, gender, BMI, plasma calcium and phosphorus levels, cardiovascular risk factors (diabetes, hypertension), use of angiotensin-converting enzyme inhibitors (ACEI)/angiotensin receptor blockers (ARB), creatinine clearance, hs-CRP levels and augmentation index (Ngo, D.T. et al. 2010). In addition, a study conducted by Jablonski et al (Jablonski et al. 2011), showed that endothelial cells obtained from antecubital vein in vitamin D deficient middle age and older adults expressed higher levels of NF κB when compared to a parallel vitamin D sufficient group. Talmor et al (Talmor et al. 2008a), have also shown that treatment with an active vitamin D_3 metabolite, calcitriol suppressed lipopolysaccharide induced DNA-binding activity of NF κB confirming the anti-inflammatory effects of vitamin D (Talmor et al. 2008a). It has been suggested that 1,25-dihydroxyvitamin D_3 negatively regulates interleukin 12 by down regulating nuclear factor κB (NF-κB) activation due to the binding to the promoter region of the responsible gene (D'Ambrosio et al. 1998).

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1.2.5.2.3 Effects of vitamin D_3 on oxidative stress

Recently, studies have shown links between low vitamin D levels and oxidative stress. These studies have demonstrated the role of vitamin D in regulating oxidative stress. Specifically, Vitamin D_3 is a negative regulator of renin synthesis (Pilz et al. 2009). A reduction in renin synthesis would indeed lower angiotensin II concentrations (Deinum & Chaturvedi 2002),

and angiotensin II, a major source of superoxide production, has been suggested as a major contributor to the onset of Type 2 DM (Olivares-Reyes, Arellano-Plancarte & Castillo-Hernandez 2009), and endothelial dysfunction (Oak & Cai 2007). Li et al (Li et al. 2002) demonstrated that $1,25(OH)_2D_3$ suppresses renin biosynthesis. In VDR null mice, renin expression and plasma angiotensin II levels are elevated (Li et al. 2002); corresponding to an increase in water and sodium retention and development of hypertension in these mice. In line with the above findings, growing male rats fed with a vitamin D deficient diet demonstrated increased aortic superoxide production, and development of hypertension, reversible with vitamin D supplementation (Argacha et al. 2011).

Clinically, studies have demonstrated positive effects of vitamin D_3 treatment on reduced oxidative stress and inflammation. The administration of VDR agonists such as calcitriol or paricalcitol to hemodialysis patients significantly reduced oxidative stress after 4 weeks and 3 months of supplementation, respectively (Izquierdo, M.J. et al. 2012; Wu, CC et al. 2011). In hemodialysis patients with secondary hyperparathyroidism, 4 month (16-week) treatment with calcitriol (1 µg of calcitriol 3 times per week for 16 weeks) reduced inflammatory markers (CRP and interleukin-6 (IL-6)) and oxidative stress conditions (total oxidant status (TAS), CD4 (+) T-lymphocyte intracellular cytokines (interferon γ (IFN-γ) and IL-4) (Wu, C-C et al. 2011). In another study, patients with renal disease undergoing hemodialysis received paricalcitol treatment for 3 months which reduced the oxidative stress marker malondialdehyde (MDA) and inflammatory markers such as serum CRP,TNF-α, IL-6 and IL-18. In addition, the treatment with paricalcitrol also significantly increased antioxidants such as superoxide dismutase (SOD), thioredoxin, catalase and anti-inflammatory cytokine IL-10 levels in these patients with renal insufficiency (Izquierdo, María J et al. 2012).

1.2.5.2.4 Effects of vitamin D_3 on endothelial function

Endothelial dysfunction

The vascular endothelium consists of a layer of endothelial cells that is the interface of vessel walls and plays a key role in maintaining vascular tone by synthesizing and releasing vasoactive substances. Endothelial cells are susceptible to changes in hemodynamic forces and blood composition and respond to various biochemical stimuli. The well-described

endothelium-derived relaxing factors (EDRFs) are nitric oxide and prostacyclin, which are involved in vascular smooth muscle relaxation. The endothelium also releases substances which cause vascular contraction, known as endothelium-derived contracting factors (EDCFs), such as metabolites of arachidonic acid. The balance between endothelium-derived relaxing and contracting factors ensure the maintenance of vascular homeostasis. It is an imbalance between the vasodilating and vasoconstricting which leads to endothelial dysfunction (Deanfield et al. 2005; Poredoš 2001).

Nitric oxide (NO) is a potent endogenous vasodilator synthesized from L-arginine by endothelial nitric oxide synthase (eNOS). Nitric oxide exerts its vasodilatory effects via the stimulation of cGMP formation through the activation of guanylyl cyclase (GC) (Moncada, Palmer & Higgs 1991). Endothelium-derived NO plays a critical role in the regulation of vasodilatation, inhibition of vascular smooth muscle proliferation, prevention of adhesion of circulating leukocytes, inhibition of platelet aggregation and formation of thrombus. The reduction of bio-available NO could occur by: 1) decreased eNOS activity (Endemann & Schiffrin 2004; Sanchez et al. 2006); 2) insufficient substrate (L-arginine) or co-factors (reduced form of tetrahydrobiopterin or BH_4) for eNOS (Creager et al. 1992; Vásquez-Vivar et al. 1998); 3) increased levels of asymmetric dimethylarginine (ADMA), the competitive eNOS inhibitor (Böger, R.H. et al. 1998; Cooke, JP 2000); and 4) increased degradation rate of NO by increased inflammation (Lahera et al. 2007; Mezzano et al. 2001) or oxidative stress (Cai & Harrison 2000; Hink et al. 2001). Endothelial dysfunction as a result of decreased bio-available NO has been suggested to be one of the main mechanisms responsible for the development and progression of atherosclerosis (Cooke, JP 2000; Moncada et al. 1991).

Asymmetric dimethylarginine (ADMA) and endothelial dysfunction

Asymmetric dimethylarginine (ADMA) has been characterized as an endogenous, competitive inhibitor of nitric oxide synthase (NOS) (Miyazaki et al. 1999). Vallance et al (Leone et al. 1992) were the first to report that L-NMMA and ADMA but not SDMA, act as endogenous inhibitors of eNOS. ADMA inhibits vascular NO production in pathophysiological concentrations by competitive interaction with L-arginine for NOS.

Dimethylarginine dimethylaminohydrolase (DDAH) is the enzyme involved in hydrolytic degradation of the ADMA and L-NMMA to citrulline and methylamines. A small percentage

of ADMA and almost all of SDMA is eliminated by renal excretion (Kielstein, J.T. et al. 2006). MacAllister et al (MacAllister et al. 1996) studied the functional role for DDAH and methylarginines in the regulation of activity of NOS and demonstrated the presence of functionally active DDAH in human endothelial cells and blood vessels, indicating a role for this enzyme in the regulation of NO synthesis. The study showed that inhibition of DDAH caused an accumulation of intracellular ADMA and L-NMMA and thereby indirectly inhibited the activities of NOS. However, concentrations of SDMA were unaffected by the inhibition of DDAH, proving that SDMA is not a substrate for DDAH. They also found that inhibition of DDAH causes the gradual vasoconstriction of vascular segments, which was reversed by L-arginine. DDAH activity in endothelial cells is decreased by oxidized low-density lipoprotein (LDL) and tumor necrosis factor-α. This reduction in DDAH activity was also found in rabbits fed with a high-cholesterol diet (Ito, A. et al. 1999). Hyperglycemia-induced oxidative stress can impair the normal activities of DDAH, which leads to an elevation of serum ADMA levels and inhibition of endothelium-derived synthesis of NO (Lin et al. 2002).

ADMA inhibits vascular NO production in pathophysiological concentrations by competitive interaction with L-arginine at the level of NOS (Böger, R.H. 2003). Indeed, ADMA has been shown to cause local vasoconstriction when it is infused intra-arterially (Calver et al. 1993). Furthermore, it has been shown that increased ADMA levels are associated with impaired endothelium-dependent vasodilation (Böger, R.H. 2006; Böger, R.H. et al. 1998). Additionally, other studies have strong associations between raised ADMA levels and endothelial dysfunction (Matsuoka, H et al. 1997; Miyazaki et al. 1999; Weis et al. 2001). ADMA inhibits vascular NO production in pathophysiological concentrations by competitive interaction with L-arginine at the level of NOS. It has also been shown that increases in ADMA concentrations significantly increases angiotensin II concentrations (Veresh et al. 2012), and therefore can cause vasoconstriction.

Clinically, elevated ADMA concentrations have been suggested to be a potent biomarker of increased cardiovascular mortality and morbidity (Böger, R.H. 2003, 2006). A small increase in ADMA concentrations of 0.2 µmol/L is associated with at least a 2-fold increase in cardiovascular mortality and morbidity (Schnabel et al. 2005). Increased plasma ADMA levels have been found in patients with hypercholesterolemia (Böger, Rainer H et al. 2000), atherosclerosis (Eid et al. 2003), hypertension (Ito, Akira et al. 2001; Surdacki et al. 1999),

chronic renal failure (Kielstein, Jan T et al. 2002; Leone et al. 1992), diabetes mellitus (Lin et al. 2002) and pulmonary hypertension (Gorenflo et al. 2001; Pullamsetti et al. 2005).

Effects of vitamin D_3 on endothelial function and asymmetric dimethylarginine (ADMA) levels

Recently, in the last 10 years, it has been increasingly suggested that vitamin D is important in cardiovascular homeostasis (Lee, J.H. et al. 2008; Temmerman 2011; Wang et al. 2012; Wang, T.J. et al. 2008), and conversely, that vitamin D deficiency may represent a risk factor for the development of coronary disease (Szeto et al. 2012; Zittermann, A. & Koerfer 2008). Vitamin D levels have been correlated with various cardiovascular risk factors and events (Muscogiuri et al. 2012). One possible mechanism for these observations could be the effect of vitamin D on endothelial function (Ngo, D.T. et al. 2010; Sugden et al. 2008; Tarcin et al. 2009).

A number of previous experimental findings raise the possibility that vitamin D may increase the activity and expression of eNOS (or nitric oxide synthase III), the enzyme critical to the generation and bioavailability of NO. For example, treatment of endothelial cells with calcitriol significantly reversed advanced glycation end product-induced down-regulation of endothelial NO synthase mRNA and activity (Talmor et al. 2008b); aortic eNOS expression and urinary nitrate/nitrite excretion were reduced in vitamin D receptor knockout mice (Aihara et al. 2004). An animal study carried out in spontaneously hypertensive rats found that chronic treatment with active vitamin D metabolite reduced the endothelium-dependent vascular contractions in the aorta, resulting in reduction of blood pressure, basal reactive oxygen species and COX-1 expression (Wong et al. 2010). Furthermore, VDR expression and 1α-hydroxylase mRNA and protein expressions have been described in endothelial cells (Zehnder et al. 2002). Collectively, these results indicate a role of vitamin D in regulating NO bioavailability in endothelial cells.

Clinically, it was demonstrated in an ageing population (n=254), that low $25(OH)D_3$ levels are associated with increased plasma concentrations of ADMA (Ngo, D.T. et al. 2010), a physiologically occurring competitive antagonist of eNOS, which is both a strong marker and also a mediator of many aspects of endothelial dysfunction (Boger 2003; Boger et al. 1997; Sydow & Munzel 2003). This relationship persisted even after adjustments for season, age, body mass index (BMI) and other cardiovascular risk factors (Ngo, D. T. et al. 2010). In

addition, a study carried out in healthy middle-aged and older adults, Jablonski et al (Jablonski et al. 2011) found that FMD was lower in vitamin D-insufficient and vitamin D-deficient participants versus vitamin D-sufficient subjects. More importantly, serum 25-hydroxyvitamin D (25(OH)D) levels were positively correlated to values of FMD in this study cohort. Furthermore, this study demonstrated that lower 25(OH)D levels were associated with increased endothelial cell expression of pro-inflammatory cytokines NF κB and IL-6.

More importantly, several studies have demonstrated the effects of vitamin D supplementation on vascular function (Sugden et al. 2008; Tarcin et al. 2009). Sugden et al (Sugden et al. 2008) assessed the improvement of endothelial function by ergocalciferol (vitamin D_2) replacement in patients with Type 2 diabetes mellitus and low levels of serum 25(OH) D (< 50 nmol/L) in a placebo-controlled randomized trial. In that study, authors found that a single high dose of vitamin D_2 (100,000 IU vitamin D_2) increased 25(OH) D levels by 15.3 nmol/L relative to those of the placebo and improved FMD of the brachial artery by 2.3% compared with the placebo group. The improvement in FMD was independent from the changes in blood pressure. Another study (Tarcin et al. 2009) demonstrated significantly lower FMD values in 23 asymptomatic vitamin D deficient (25(OH) D < 25nmol/L) subjects compared to an age- and sex-matched control group (25(OH) D > 75nmol/L). After vitamin D replacement therapy of 300 000 IU of vitamin D_3 monthly for 3 months, FMD values of vitamin D deficient group was significantly increased from the baseline values. At the baseline of the study, thiobarbituric acid reactants (TBARs) levels, a marker of lipid peroxidation, were not different between the deficient group and the control group, TBARs levels decreased significantly after the vitamin D replacement for 3 months. Harris et al (Harris et al. 2011) demonstrated similar significant improvement of FMD in healthy overweight African American adults after 16 weeks of 60 000 IU monthly supplement of oral vitamin D_3 when compared to placebo treated group. Furthermore, this study reported an increased absolute change in the diameter of the brachial artery and FMD/shear ratio after the vitamin D_3 supplementation whereas no change was observed with a placebo.

1.2.5.2.5 Possible link between platelet-derived thrombospondin-1 (TSP-1) with vitamin D_3

Role of TSP-1 on regulating nitric oxide signalling

Thrombospondin-1 (TSP-1) is a matricellular glycoprotein that is transiently expressed in the extracellular matrix and regulates cell function through surface receptors and other components of the extracellular matrix (Bornstein, Paul 1995). The term "matricellular" refers to matrix proteins with a modular structure that do not play a part in vascular wall structural integrity (Bornstein, Paul 2001). TSP-1 is large, trimetric, glycoprotein composed of three identical disulfide-linked peptide chains (Coligan & Slayter 1984). Normal plasma contains low but significant levels of circulating TSP-1 at approximately 100-200 pM (Bergseth et al. 2000). Platelet α-granules is the major pre-formed storage of TSP-1 and extracellular concentration of the protein can be rapidly increased at the sites of injuries with the activation of platelets (Roberts, David D et al. 2012). TSP-1 is also secreted by a wide variety of epithelial and mesenchymal cells including endothelial, vascular smooth muscle cells (VSMC) and kidney mesangial cells (Bornstein, P 1992).

In addition, TSP-1 has been shown to be a negative regulator of NO signaling. Regarding NO's effect on vascular endothelium, NO has a biphasic effect on endothelial cell proliferation and angiogenesis. The cGMP pathway is activated by the lowest doses of NO by activating soluble guanylyl cyclase (sGC) to increase cGMP which activates cGMP-dependent protein kinases and other cGMP-dependent pathways and mediates stimulatory effects of NO on endothelial cell adhesion, and proliferation (Kawasaki et al. 2003; Morbidelli et al. 2003; Zaragoza et al. 2002). cGMP signaling plays a central role in both acute responses to NO and long-term proliferation responses. TSP-1 has been shown to be an inhibitor of soluble guanylate cyclase (sGC) by blocking both haeme-dependent and haeme-independent activation of sGC and thus NO-cGMP signaling (Isenberg, Jeff S et al. 2005; Isenberg, Jeff S. et al. 2008; Miller, TW, Isenberg & Roberts 2010). TSP-1 null murine platelets failed to aggregate in response to NO/cGMP signal. At physiological concentrations of NO, TSP-1 null platelets showed elevated levels of cGMP. Small concentrations of TSP-1 have been reported to inhibit NO-stimulated activation of sGC in platelets (Isenberg, Jeff S. et al. 2008). These findings emphasized the inhibitory activity of TSP-1 on NO/cGMP signalling.

CD47 and CD36 have been described to be receptors for TSP-1 activity. These are integral membrane proteins which are expressed in vascular and circulating blood cells. TSP-1 signalling activities were shown to be lost in CD47 null mice but not in CD36 null mice (Isenberg, Jeff S. et al. 2006), indicating the necessity of CD47 as the primary receptor to regulate TSP-1 activity. The interaction of TSP-1 with CD36 also inhibits NO/cGMP signalling, but at higher concentrations of TSP-1 and in only the cells that express the CD47 receptors. Furthermore, TSP-1 is reported to inhibit physiological activity of eNOS by interrupting the negative regulatory activity of caveolin-1 (Ju et al. 1997) on eNOS. In endothelial cells CD47 is constitutively associated with caveolin-1 to regulate eNOS activity; binding of TSP-1 to CD47 receptor disrupts this constitutive association leading to eNOS hyperactivity and superoxide radical generation. This mechanism was postulated for explaining association between elevated TSP-1 levels and pulmonary arterial hypertension (Bauer, PM et al. 2012).

Consequences of elevated TSP-1 levels

Increased levels of TSP-1 have been associated with several diseases including atherosclerosis, hypercholesterolemia, myocardial infarction and diabetes (Riessen et al. 1998; Roth et al. 1998; Sezaki et al. 2005; Varma et al. 2008), and inhibition of NO/cGMP signalling has been identified as a cause for this association.

A study carried out with TSP-1 null mice demonstrated that TSP-1 prevented NO-mediated relaxation of pre-contracted vascular smooth muscle cells. Using blood oxygen level-dependent (BOLD) magnetic resonance imaging (MRI), which allows non-invasive assessment of tissue blood flow in resting animals, the authors found that following a NO challenged (by nitric oxide donor DEA/NO) TSP-1 null mice showed a 2-fold greater increase in skeletal muscle blood flow compared to wild type controls. These results demonstrate that endogenous TSP-1 constantly limits NO signaling to control regional blood flow under resting conditions (Isenberg, Jeff S. et al. 2007a).

Endogenous TSP-1 limits tissue survival and recovery of oxygenation following ischemic injury through limiting the angiogenic and vasodilator activities of NO (Isenberg, Jeff S. et al. 2007a). A study showed that CD47-null mice demonstrated lesser tissue necrosis in response to fixed ischemic injury compared to wild-type mice that were exposed to the same ischemic

challenge. The functional blockage of CD47 receptor increases ischemic tissue survival of these wild-type mice (Isenberg, Jeff S. et al. 2007b). Therapeutic intervention studies targeting TSP-1 and its receptor CD47 may improve tissue survival with the potential to overcome ischemic tissue necrosis.

Elevated plasma TSP-1 concentrations have been found in stroke (Jurk, K. et al. 2010; Ning et al. 2011), peripheral arterial disease (Smadja, D. M. et al. 2011), and myocardial ischemia (Frangogiannis, N. G. et al. 2005), all of which are conditions associated with increased thrombogenicity and impaired nitric oxide signaling. A study carried out in a canine model of reperfused infarction showed that TSP-1 mRNA expression was induced after the ischemia and reperfusion (Frangogiannis, Nikolaos G et al. 2005). Another study carried out in patients with carotid artery stenosis demonstrated that significantly enhanced surface expression of TSP-1 levels in the platelets relative to an age-matched healthy control group (Jurk, Kerstin et al. 2010). Furthermore, plasma TSP-1 levels have been shown to be linearly associated with peripheral arterial disease (PAD). In this study, patients with PAD showed significantly increased plasma levels of TSP-1 relative to aged-matched controls (Smadja, David M et al. 2011).

Association of TSP-1 levels with vitamin D_3

There is evidence to suggest that vitamin D is a regulator of TSP-1. In human aortic smooth muscle cells, the addition of vitamin D analogs calcitriol and paricalcitol significantly decreased TSP-1 mRNA and protein expression. In this study, TSP-1 mRNA levels were reduced in a dose-dependent manner and maximum suppression was detected at 0.1µM concentration of the analogs (Wu-Wong, J R, Nakane, M & Ma, J 2006). It has been suggested that platelet-derived TSP-1 functions with D-binding protein (DBP) to enhance the chemotactic activity by acting as an additional chemotactic co-factor to complement component 5a (C5a) (Trujillo & Kew 2004) and therefore exerts anti-inflammatory effects.

1.2.5.2.6 Possible link between vitamin D_3 and platelet nitric oxide responsiveness

The vascular endothelium derived NO modulates the homeostatic interaction between platelets and the vessel wall. The inhibition of platelet aggregation by NO is mediated by the

stimulation of soluble guanylate cyclase (sGC) and followed by a rise in cyclic guanosine monophosphate (cGMP) (Furlong et al. 1987; Mellion et al. 1981).

Studies have demonstrated a decreased platelet responsiveness to NO as a result of the decreased sensitivity of guanylate cyclase to NO and the inactivation of released NO by superoxide radicals (O_2^-) in aggregating platelets of patients with stable angina pectoris with respect to normal subjects (Chirkov, Y.Y. et al. 1999). It has been documented that increased levels of superoxide radicals (O_2^-) in oxidative stress cause NO resistance which results from a combination of inactivation of NO by superoxide anion radicals and of (reversible) inactivation of soluble guanylate cyclase (sGC) by superoxide anion radical through alterations of the redox state of heme moiety of sGC, which is the target for NO. The impaired responsiveness of platelets to exogenous NO donors, such as sodium nitroprusside (SNP), *in vitro* implies a reduced platelet sensitivity to endogenous NO *in vivo*, resulting in platelet hyperaggregability. Thus, platelet hyperaggregability and the impaired NO responsiveness may contribute to the increased risk of ischemic events (Chirkov, Y.Y. & Horowitz 2007; Willoughby et al. 2005). A study carried out in 76 patients with diabetes mellitus and acute coronary syndromes demonstrated that hyperglycemia correlated with increased superoxide radical generation and impaired responsiveness of the platelets to SNP. This may be due to increased NO clearance by superoxide radicals. The authors showed reduced superoxide radical generation ($p = 0.049$) and improved whole blood platelet sodium nitroprusside responsiveness ($p < 0.001$) in patients who received intravenous insulin therapy when compared with patients who received subcutaneous insulin (Worthley et al. 2007). Furthermore, a number of other agents such as ACE inhibitors, perhexiline and statins have been shown to improve the impaired NO response (Chirkov, Y.Y. & Horowitz 2007).

Vitamin D supplementation has been shown to exert effects on the nitric oxide, inflammatory and oxidative systems (Schleithoff et al. 2006),(Zia et al. 2011) in various health conditions. It is entirely possible, that low vitamin D status is associated with impaired platelet NO responsiveness, and therefore exerts detrimental effects on the cardiovascular system. To date, no study has examined the effects of vitamin D on platelet NO responsiveness.

1.2.5.2.7 Regulation of vascular calcification

Higher calcium levels in the blood have been associated with higher vascular calcification and cardiovascular events (Bolland, M J et al. 2008; Bolland, M J et al. 2011). Vascular calcification is a common risk factor in atherosclerosis, arterial stiffness and myocardial infarction. Furthermore, there is a regulatory balance between vitamin D levels and vascular calcification. Very high vitamin D concentrations have been associated with significant increases in vascular calcification (Jono et al. 1998; Mizobuchi et al. 2007). VDR activators, such as calcitriol and paricalcitol, were protective against aortic calcification at the dosages sufficient to correct secondary hyperparathyroidism, but higher dosages of these VDR activators stimulated the aortic calcification in a mouse model of chronic kidney disease (CKD) (Mathew et al. 2008). However, studies have also shown that circulating vitamin D levels that are below the physiological range, i.e. vitamin D deficient status, are associated with vascular calcification (Mathew et al. 2008; Raggi et al. 2011; Watson et al. 1997). A study by de Boer et al (de Boer et al. 2009) from the Multi-Ethnic Study of Atherosclerosis with 1370 participants free of coronary artery calcification (CAC) at baseline, demonstrated that the lower $25(OH)D_3$ level was associated with an increased risk of developing coronary artery calcification over 3 years of follow up. Specifically, each 10 ng/ml lower $25(OH)D_3$ concentration was associated with a 23% increased risk for developing (CAC) after adjustment for age gender, race/ethnicity, or serum phosphate concentration ($p=0.049$). Furthermore, the circulating concentration of $1,25(OH)_2D_3$ has been shown to be inversely associated with vascular calcification. This study demonstrated an inverse correlation between vascular calcification and active metabolite of vitamin D_3 ($1,25(OH)_2D_3$) levels in subjects at high and moderate risk for coronary heart disease (Watson et al. 1997).

1,25-dihydroxyvitamin D_3 reduces vascular calcification by increasing Matrix Gla protein mRNA expression. Vitamin D_3 has been shown to increase MGP mRNA expression in human osteoclasts and rat chondrocytes, osteoblasts and osteosarcoma cells (Proudfoot & Shanahan 2006). Furthermore, studies demonstrated the dramatic increase in the expression of MGP mRNA by 1,25-dihydroxyvitamin D_3 treatment to clonal rat osteosarcocmelal lines (Fraser, Otawara & Price 1988).

Matrix Gla protein (MGP) was originally isolated from bone but it is known to be expressed in several tissues including kidney, lung, heart, cartilage and vascular smooth muscle cells (VSMC) of the blood vessel wall. It is an 84-amino-acid protein with molecular weight of

approximately 12 kDa that contains five γ–carboxyglutamic acid (Gla) residues. The Gla residues in MGP and all other vitamin K-dependent proteins are produced by γ-carboxylation of certain glutamic acid residues by γ-carboxylase, and require a reduced form of vitamin K as a cofactor. The only known function of Gla residues is to bind calcium ions or calcium crystals. MGP is a calcification-inhibitor and regulates calcium deposition in several ways (Proudfoot & Shanahan 2006):

(i) Binding calcium ions and crystals;
(ii) Antagonizing bone morphogenetic protein and altering cell differentiation;
(iii) Binding to extracellular matrix components and;
(iv) Regulating apoptosis.

Therefore, the active form of vitamin D, $1,25(OH)_2D_3$, is essential to maintain optimal physiological level of calcium and equally it is important to prevent vascular calcification at higher calcium levels in the body.

1.2.5.3 Cardiovascular consequences of vitamin D deficiency

In the past 10 years, it has increasingly been suggested that vitamin D is important in cardiovascular homeostasis (Lee, J.H. et al. 2008; Temmerman 2011; Wang et al. 2012; Wang, T.J. et al. 2008), and conversely, that vitamin D deficiency may represent a risk factor for the development of coronary disease (Szeto et al. 2012; Zittermann, A. & Koerfer 2008). Scragg et al (Scragg, R. et al. 1990) first reported that in the community, an inverse relationship occurred between serum $25(OH)D_3$ levels and myocardial infarction (MI). In this case-control study of 179 patients, MI patients had significantly lower 25-hydroxyvitamin D levels than the controls (32 vs 35.5 nmol/L; p=0.017). Martins et al (Martins et al. 2007b) demonstrated inverse associations between vitamin D levels and incidence of obesity, hypertension and diabetes. The Framingham offspring study (Wang, T. J. et al. 2008) found that vitamin D deficiency is associated with increased risk of cardiovascular events irrespective of pre-existing cardiovascular risk factors. Furthermore, low vitamin D status has been found to be associated with increased risk of myocardial infarction independent of conventional cardiovascular risk factors (Giovannucci et al. 2008b); as well as all-cause and cardiovascular mortality (Dobnig, Harald et al. 2008; Dobnig, H. et al. 2008a; Naesgaard et al. 2012; Schierbeck et al. 2011; Tomson, J. et al. 2013; Zhao et al. 2012).

In another study of 18,225 males from the Health Professionals Follow-Up study (Giovannucci et al 2008) who did not have a history of cardiovascular diseases, vitamin D levels of lower than 15ng/ml (37.5 nmol/L) were associated with approximately 2 and a half fold increase in the risk of MI when compared to those with a level of >30ng/ml (75 nmol/L). A similar result was also found from the 1739 Framingham Offspring Study patients without prior coronary heart disease (Wang et al 2008) (Wang, T.J. et al. 2008), although the association was only found in hypertensive patients and not on non-hypertensive patients. Many other epidemiological studies also confirmed the association between hypovitaminosis D and cardiovascular events, either in patients with coronary artery disease (Dobnig et al 2008 (Dobnig, Harald et al. 2008); Pilz et al 2008 (Pilz et al. 2008); Karakas et al, (Karakas et al. 2013) 2013), heart failure (Holick et al 2007 (Holick, M.F. 2007); Anderson et al 2010 (Anderson, Jeffrey L. et al. 2010)), diabetes (Pittas et al 2007(Pittas, A.G. et al. 2007); Afzal et al 2012 (Afzal, Bojesen & Nordestgaard 2012); Xuan et al 2013 (Xuan, Zhao & Liu 2013)) or renal diseases (Wolf et al 2007 (Wolf et al. 2007); Wang et al 2008 (Wang, T.J. et al. 2008); Ravani et al 2008 (Ravani et al. 2008)). The emergence of these epidemiological findings of the potential importance of vitamin D status to cardiovascular disease/outcomes is apparent, but mechanistic insights of these findings remain largely elusive. The summary of these studies are presented in Table 1.2.

Table 1.2: Cardiovascular consequences of vitamin D deficiency

Authors	Study participants	Follow-up period	Key findings
Wolf et al (Wolf et al. 2007)	825 patients with ESRD from 569 haemodialysis centres	90 days	• Female, black or diabetic subjects were more likely to be severely vitamin D deficient (<10ng/ml) • Subjects with $25(OH)D_3$ < 10 ng/ml were at higher risk of all-cause mortality than > 30 ng/ml
Giovannucci et al (Giovannucci et al. 2008a)	18225 males (age 40-75 years) with no previous CVD from Health Professionals Follow-Up study;	10 years	• Vitamin D status of <15ng/ml increases risk of MI when compared to vitamin D status of >30ng/ml (Relative risk 2.42)
Wang et al (Wang, T.J. et al. 2008)	1739 white participants (age 59 years) without prior CVD from Framingham Offspring Study (55% female); age	5.4 years	• Hypertensive participants with $25(OH)D_3$ < 15 ng/ml had higher incident of CV events compared to those with $25(OH)D_3$ > 15 ng/ml. • This effect was evident in hypertensive participantsbut not in normotensives
Dobnig et al (Dobnig, Harald et al. 2008)	3258 white patients (age 62 years) referred for coronary angiography in a single centre;	7.7 years	• Low $25(OH)D_3$ and $1,25(OH)_2D_3$ associated with all-cause and cardiovascular mortality • Low $25(OH)D_3$ levels correlated with inflammation, oxidative burden and cell adhesion
Melamed et al (Melamed et al. 2008)	13,331 adults > 20 yearsfrom NHANES III study	8.7 years	• Vitamin D < 17.8 ng/ml is associated with 26% increase of all-cause mortality
Pilz et al (Pilz et al. 2008)	3299 Caucasians referred for coronary angiography	7.7 years	• $25(OH)D_3$ and $1,25(OH)D_3$ are inversely correlated with NT-proBNP and left ventricular function • NYHA class inversely correlated with vitamin D status
Wang et al (Wang, AY-M et al. 2008)	230 patients with ESRD undergoing peritoneal dialysis	3 years	• Increased in fatal or non-fatal cardiovascular events in $25(OH)D_3$ <45.7nmol/L, in patients with preserved left ventricular systolic function or less left ventricular hypertrophy

Authors	Study participants	Follow-up period	Key findings
Ravani et al (Ravani et al. 2008)	168 patients with stage 2 to 5 chronic kidney disease	4 years	• $25(OH)D_3$ is independent inverse predictor of disease progression/death
Joergensen et al (Joergensen et al. 2010)	289 patients with Type 2 DM from a tertiary referral centre	15 years	• Severe vitamin D deficiency (<13.9nmol/L) associated with cardiovascular and all-cause mortality in diabetic patients, independent of baseline renal function and duration of diabetes
Anderson et al (Anderson, Jeffrey L. et al. 2010)	Electronic medical records database contained 41,504 patient records with at least one measured vitamin D level	Not given	• Vitamin D deficiency associated with increases in the prevalence of diabetes, hypertension, hyperlipidemia, and peripheral vascular disease ($p<0.0001$) • Vitamin D levels correlated with coronary artery disease, myocardial infarction, heart failure and stroke ($p<0.0001$)
Joergensen et al (Joergensen et al. 2010)	289 type 2 diabetes mellitus patients with normal urinary albumin excretion at baseline.	15 years (0.2-23 yrs)	• All-cause mortality was increased in patients with severe vitamin D deficiency ($25(OH)D_3$ <13.9 nmol/L) independent from urinary albumin excretion, diabetes and cardiovascular risk factors • Severe vitamin D deficiency independently associated with increased cardiovascular mortality
Afzal et al (Afzal, Bojesen & Nordestgaard 2012)	9841 participants from general population free from diabetes at baseline	29 years	• Low $25(OH)D_3$ levels associated with higher incidence of type 2 diabetes
Brondum-Jacobsen et al (Brøndum-Jacobsen et al. 2012)	18,791 participants from the Copenhagen City Heart Study and the Copenhagen General Population Study	30 years	• Stepwise increasing risk of venous thromboembolism with decreasing tertiles of seasonally adjusted plasma 25-hydroxyvitamin D concentrations
Wang et al (Wang et al. 2013)	1,211 US men that were free of baseline hypertension	15.3 years	• Plasma $25(OH)D_3$ levels inversely associated with risk of hypertension
Brondum-Jacobsen et al (Brøndum-Jacobsen et al. 2013)	10,170 individuals from the general population, the Copenhagen City Heart Study	21 years	• Stepwise increasing risk of symptomatic ischemic stroke with decreasing plasma 25-hydroxyvitamin D concentrations

Authors	Study participants	Follow-up period	Key findings
Skaaby et al (Skaaby et al. 2012)	9,146 individuals from the two population-based studies, Monica10 and Inter99	10 years	• Inverse association was reported between serum vitamin D status and all-cause mortality
Schierbeck et al (Schierbeck et al. 2012)	2016 healthy, recently postmenopausal women who were enrolled in the Danish Osteoporosis Prevention Study	16 years	• Women with vitamin D deficiency had more cardiovascular risk factors than vitamin D-replete women • Healthy women with vitamin D deficiency have increased risk of adverse cardiovascular outcome
Fall et al (Fall et al. 2012)	870 participants (aged 70 years) (52% women) without prior myocardial infarctions, heart failure, or prevalent valvular disease	5 years	• Higher circulating $25(OH)D_3$ levels associated cross-sectionally with better LV systolic function and smaller LVESD at baseline
Tomson et al (Tomson, Joseph et al. 2012)	5409 of older men living in the UK (Whitehall study)	13 years	• Higher concentrations of $25(OH)D_3$ inversely associated with vascular and non-vascular mortality throughout the range of 40-90 nmol/L independent from age and seasonality • Doubling in $25(OH)D_3$ concentration was associated with 20% [95% CI: 9–30%] lower vascular and 23% (95% CI: 14–31%) lower non-vascular mortality
Karakas et al (Karakas et al. 2013)	1783 healthy middle-aged subjects in the population-based Monitoring of Trends and Determinants in Cardiovascular Disease in MONICA/KORA Augsburg Case-Cohort Study	11 years	• Higher vitamin D levels are associated with decreased risk of coronary heart diseaseand is more pronounced in women than in men

Abbreviations: CVD = Cardiovascular disease; FHx = Family history; MI = Myocardial infarction; BMI = Body mass index; LDL = Low density lipoprotein; HDL = High density lipoprotein; TG = Triglycerides; CRP = C-reactive protein; SeCr = Serum creatinine; eGFR = estimated glomerular filtration rate; TIA = Transient ischaemic attack; HF = Heart failure; NYHA = New York Heart Association; HbA1c = Glycosylated haemoglobin; HTN = Hypertension; NHANES = ; PTH = Parathyroid hormone; ESRD = End stage renal disease; $25(OH)D_3$ = 25-hydroxyvitamin D; $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3; LVESD = left ventricular end-systolic diameter.

However, to date there is a limited amount of evidence from randomized trials (RT) on the effects of vitamin D supplementation alone vs a placebo to examine cardiovascular benefits. A placebo-controlled randomized trial carried out with congestive heart failure (CHF) patients demonstrated vitamin D supplementation of 50 μg (2000 IU) vitamin D_3 daily for 9 months significantly increased anti-inflammatory cytokine, interleukin 10 levels by 43% in the treatment group compared to the baseline (p=0.035) (Schleithoff et al. 2006). The pro-inflammatory cytokine, tumor necrosis factor α (TNFα) was increased in the placebo group but remained constant in the vitamin D supplemented group after 9 months of the treatment (Schleithoff et al. 2006). Vitamin D supplementation has been reported to improve endothelial function in both vitamin D deficient healthy adults as well as in patients with cardiovascular risk factors (Harris et al. 2011; Sugden et al. 2008; Tarcin et al. 2009).

While interventional studies reported positive outcomes with vitamin D supplementation, others have also reported no effect on endothelial function or cardiovascular risk factors. A large observational study (Women's Health Initiative (WHI) randomized trial) with vitamin D supplementation, including 36,282 post-menopausal women showed no decrease in cardiovascular risk with vitamin D supplementation over the 7 years of follow up (Hsia et al. 2007). Limitations of this study were, the use of a very low vitamin D dose (400 IU/day), baseline or post-treatment vitamin D levels were not measured and vitamin D status was not considered as a factor for enrolment. In a different placebo controlled study with type 2 diabetic subjects showed no effect of vitamin D supplementation on endothelial function (Witham et al. 2010). However, this study enrolled participants with a wide range of baseline 25(OH)D_3 levels (25(OH)D_3 < 100 nmol/L), which possibly masked the effect of changes of vitamin D on cardiovascular homeostasis. The same authors (Witham, MD et al. 2012) carried out another study involving patients with previous history of stroke and vitamin D levels < 75 nmol/L at the baseline, which reported significantly higher FMD at 8 weeks in the intervention group with single dose of 100 000 IU of vitamin D_2 at the baseline compared to placebo treated group (p=0.007). However, the effect was not significantly different after 16 weeks of the treatment (Witham, M et al. 2012). Several other interventional randomized clinical trials reported no cardiovascular event difference by the treatment (Brazier et al. 2005; Prince et al. 2008; Trivedi, Doll & Khaw 2003). The summary of these studies are presented in Table 1.3.

Results from clinical trials with pre-defined cardiovascular endpoints such as myocardial infarction, stroke and cardiovascular mortality are pending. These studies will definitively

prove whether or not, vitamin D supplementation will have additional cardiovascular benefits (Al Mheid et al. 2013).

Table 1.3: Effect of vitamin D supplementation on cardiovascular system

Study	Study type (size/n)	Deficiency definition	Patient type (number of subjects)	Dose of vitamin D given	Clinical measure	Outcome
Sugden et al (Sugden et al. 2008)	Case-control (87)	<50 nmol/L (20 ng/ml)	Type 2 DM and vitamin D deficient (87)	100 000 IU single dose of vitamin D2 or placebo	FMD	FMD was improved, decreased systolic blood pressure in treatment group
Tarcin et al (Tarcin et al. 2009)	Case-control (23)	<25 nmol/L (10 ng/ml)	Asymptomatic and vitamin D deficient (23)	300 000 IU of vitamin D3 monthly for 3 months for treatment group or placebo	FMD, leptin levels, insulin sensitivity index (glucose tolerance), lipid peroxidation (TBARS)	FMD measurements were lower in deficient subjects, Improved significantly after the replacement, TBARS levels gone down after the treatment, positive correlation between $25(OH)D_3$ and FMD, leptin levels positively correlated with $25(OH)D_3$
Harris et al (Harris et al. 2011)	Case-control (57)	<50 nmol/L (20 ng/ml)	Healthy middle-aged African Americans (57)	60 000 IU of vitamin D_3 monthly or placebo for 16 weeks	FMD, shear rate, Biochemical parameters	FMD measurements improved significantly after the replacement, increased in absolute change in brachial artery diameter FMD/shear ratio
Witham et al (Witham, M et al. 2012)	Case-control (58)	Not given	Patients with stroke, community dwelling, baseline $25(OH)D_3 < 75$ nmol/L (58)	Single oral dose of 100 000 IU vitamin D_2 or placebo at baseline	FMD, change in 24 hr blood pressure, ECG, biochemical parameters	No change of blood pressure between groups, treatment group showed significantly higher FMD after 8 weeks but no different after 16 weeks of the treatment

Study	Study type (size/n)	Deficiency definition	Patient type (number of subjects)	Dose of vitamin D given	Clinical measure	Outcome
Witham et al (Witham et al. 2010)	Case-controlled (61)	Not given	Patients with type 2 diabetes and 25(OH)D < 100 nmol/L (61)	Single oral dose of 100 000 or 200 000 IU vitamin D_3 or placebo at baseline	FMD, Office blood pressure measurements	No significant difference in FMD measurements after 8 or 16 weeks of the treatment, Insulin resistant or $HbA1_C$ levels not improved, Systolic blood pressure was lowered in treatment arms than placebo after 8 weeks, BNP levels significantly lowered in 200 000 IU group by 16 weeks
Hsia et al (Hsia et al. 2007) and LaCroix et al (LaCroix et al. 2009)	Case-controlled (36 282)	Not given	Post- menopausal women (WHI randomized trial) (36 282)	400 IU of vitamin D_3 and 1000 mg of calcium or placebo daily for 7 years	Mortality by cause	No decrease in cardiovascular risk by vitamin D treatment
Trivedi et al, (Trivedi, Doll & Khaw 2003)	Case-controlled (2686)	Not given	Free living people (2686)	100 000 IU oral vitamin D_3 or placebo every four months over five years	Fracture incident or total mortality by cause	Prevent fracture, a non-significant trend (RR _ 0.84; 95% CI, 0.055–1.10) toward a reduction in CV deaths
Prince et al, (Prince et al. 2008)	Case-controlled (302)	Not given	community-dwelling ambulant older women with a serum 25-hydroxyvitamin D concentration of less than 24.0 ng/mL and a	1000 IU of vitamin D_{2+}1000 mg calcium or placebo+1000 mg calcium daily	Risk of falls, physical activities, biochemical parameters	Reported as adverse events ischemic heart disease event rates of 1.3% in those on vitamin D_2 *vs.* 2.0% for placebo (two *vs.* three events)

Study	Study type (size/n)	Deficiency definition	Patient type (number of subjects)	Dose of vitamin D given	Clinical measure	Outcome
			history of falling in the previous year (302)			
Brazier et al, (Brazier et al. 2005)	Case-controlled (289)	≤ 30 nmol/L (12 ng/ml)	Ambulatory elderly women with vitamin D insufficiency (289)	Combination tablet with 400 IU of vitamin D_3 and 500 mg calcium twice daily or placebo for 1 year	Blood pressure, grip strength, biochemical parameters	11 cardiovascular events reported, which did not differ by the treatment

Abbreviations: BNP = Brain Natriuretic peptide; CI= Confidence interval; CV= Cardiovascular; DM= Diabetes mellitus; ECG= Electrocardiogram; FMD= Flow mediated dilatation; HbA_1C= Glycosylated heamoglobin; IU= International units; RR= Relative risk; TBARS= Thiobarbituric acid-reactive substances; WHI= Women's health initiative.

1.3 Effects of elevated parathyroid hormone (PTH) on the cardiovascular system

Low serum vitamin D levels can trigger both the synthesis and release of PTH, and conversely, increased levels of vitamin D suppresses the mRNA transcription of PTH, leading to lowered PTH synthesis. This negative feed-back mechanism is explained in Section 1.2.5.1.2 (Regulation of PTH secretion). Figure 1.3 summarizes regulation of biosynthesis and metabolism of PTH.

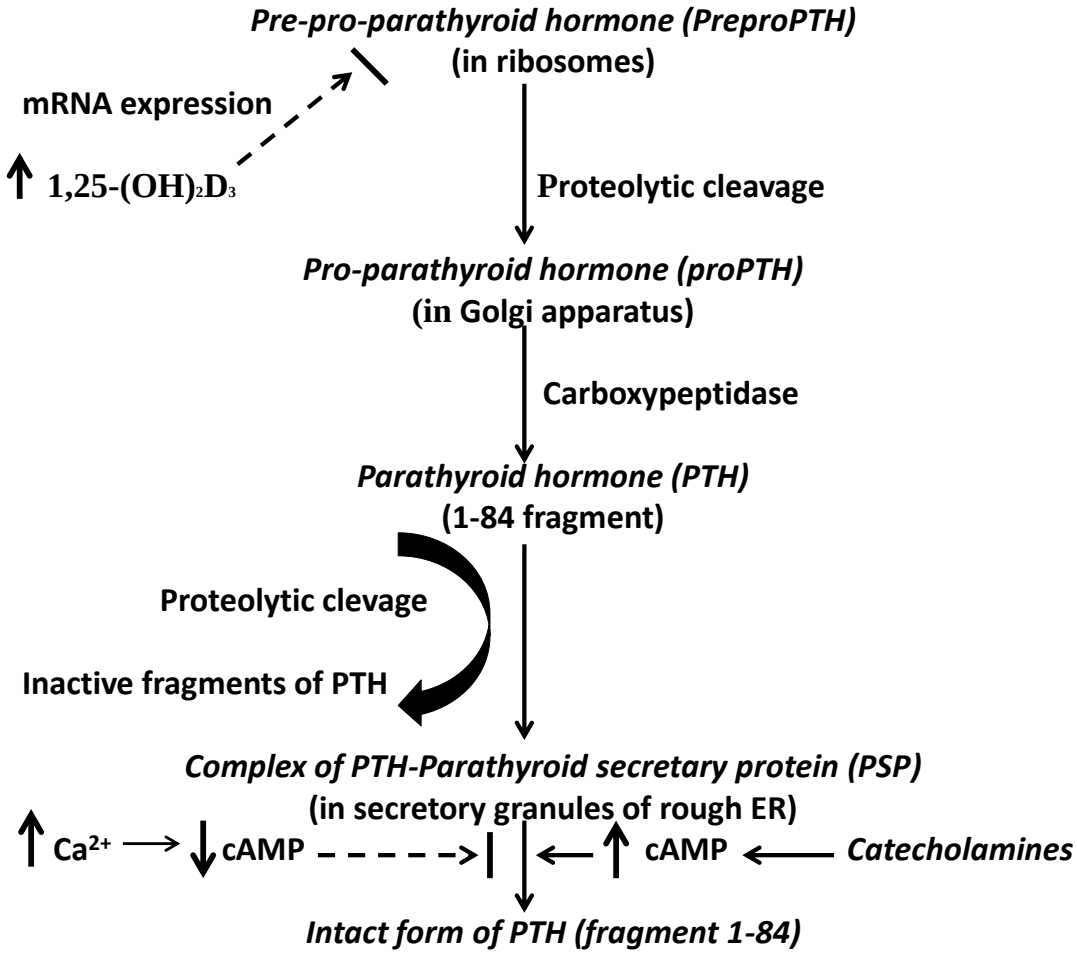

Figure 1.3 Biosynthesis and metabolism of PTH.

Effects of PTH on the cardiovascular system

Elevated PTH levels have also been linked to increased cardiovascular events. Several lines of evidence have suggested that disturbances in vitamin D and PTH metabolism have been

associated with increased cardiovascular risk through diverse pathways (Block et al. 2004; Levin et al. 2006; Pirro, M et al. 2012; Sherman, Hollis & Tobin 1990).

Elevated levels of PTH as a result of either primary hyperparathyroidism or secondary hyperparathyroidism have been found to be associated with increased cardiovascular mortality and morbidity (Block et al. 2004; Hagström et al. 2009; Palmer et al. 2011; Rostand & Drüeke 1999; Slinin, Foley & Collins 2005; Yu et al. 2010) independent of age, BMI, systolic blood pressure, diabetes, smoking, total cholesterol, HDL cholesterol, history of CVD and treatments with lipid-lowering and anti-hypertensive drugs. Plasma levels of PTH have also been reported to predict cardiovascular mortality even in the individuals with PTH levels within the normal reference levels (PTH < 6.8 pmol/L) (Hagström et al. 2009). Several lines of evidence suggest that a high PTH level is a possible cardiovascular risk factor. Elevated levels of PTH have been associated with an increased risk of hypertension (Rubin et al. 2005; Smith et al. 2000) and impaired glucose tolerance (Tassone et al. 2009) in patients with primary hyperparathyroidism (PHPT). Furthermore, patients with PHPT also have impaired insulin sensitivity (Chiu et al. 2000; Kumar et al. 1994), elevated LDL-cholesterol levels and decreased HDL-cholesterol levels (Hagström et al. 2002), and elevated circulating inflammatory markers such as hs-CRP and TNF-α levels independent of vitamin D status, and renal function (Øgard et al. 2005). In addition, PHPT patients have also been reported to have increased risk of congestive heart failure (Siu et al. 2007; Vestergaard et al. 2003). Importantly, the removal of hyperparathyroid stimuli (by parathyroidectomy) has been reported to improve cardiovascular risk (Kosch et al. 2000; Osto et al. 2012; Schillaci, Giuseppe et al. 2011; Vaziri et al. 1998), indicating that normalizing PTH status is important for cardiovascular homeostasis. However, it remains unclear what the mechanism(s) of PTH on the cardiovascular system, and whether these effects are independent from vitamin D.

1.3.1 Effects of parathyroid hormone on endothelial function

Parathyroid hormone (PTH) has been shown to exert effects on vascular endothelial function. Studies carried out with animal models and humans have both shown the possible reduction of endogenous NO production in chronic renal failure (CRF), the condition with higher circulating levels of PTH in the blood (Vaziri et al. 1998).

Clinically, an elevation in PTH levels was associated with endothelial dysfunction, although the mechanism(s) underlying this association are uncertain. Recently, Coen et al. (Coen et al. 2009), demonstrated that there was a correlation between plasma ADMA levels and PTH levels in patients with chronic renal failure who were on hemodialysis, independent of serum calcium, calcium-phosphorus product, systolic blood pressure and BMI. However, this study did not look at whether this relationship was independent of vitamin D status (Coen et al. 2009). A study carried out in patients with primary hyperparathyroidism (PHPT) reported an impairment of endothelium dependent FMD at baseline compared to the age-, sex- and blood pressure-matched healthy controls (4.7±1.2 vs 18.2±3.7%, $p<0.01$) and this improved significantly after parathyroidectomy (16.7±3.0%, $p<0.01$) (Kosch et al. 2000). Furthermore, PTH has been reported to cause large arterial stiffness (Schillaci, G. et al. 2011) in patients with PHPT. In this study, aortic pulse wave velocity (PWV), a measure of aortic stiffness was significantly higher among patients with PHPT when compared to age, sex and blood pressure matched controls (11.4±2 Vs 9.6±2 m/s, $p<0.001$) and the PWV significantly decreased after parathyroidectomy (10.9±2 to 9.8±2 m/s, $p=0.003$). In another recent study, aortic PWV was significantly higher in postmenopausal women with low vitamin D levels (25-(OH)D_3 <50 nmol/l/ 20 ng/mL) when compared to postmenopausal women with higher vitamin D levels (25-(OH)$D_3 \geq 50$ nmol/l / 20 ng/mL) (9.8±0.2 Vs 8.9±0.4, $p<0.05$). However, this negative association between vitamin D level and PWV was no longer significant when adjusted for PTH levels. PTH is reported as an independent predictor of higher PWV (and arterial stiffness) ($\beta=0.23$, $p=0.007$) and this association was proposed to explain the relationship between low vitamin D levels and arterial stiffness (Pirro, M. et al. 2012). A recent study demonstrated that PTH is an independent correlate of coronary microvascular impairment in patients with pHPT (Osto et al. 2012). In this study, PTH levels were inversely correlated with coronary flow reserve (CFR; marker of coronary microvascular function) ($r=-0.3$, $p<0.004$) which normalized after parathyroidectomy.

1.3.2 Effects of parathyroid hormone on oxidative stress and inflammation

Elevated PTH levels have been found to be associated with oxidative stress and inflammation. Chronic elevation of PTH in endothelial cells, modulates Ca^{2+} signalling pathways, which activates protein kinase C (PKC) (Throckmorton et al. 2002) associated with ROS generation (PKC induce generation of ROS and oxidative stress (Yan et al. 2006))

and impairment of eNOS activity, reduction of NO bioavailability (Virdis et al. 2010) and vascular smooth muscle contraction in patients with PHPT (Tanaka et al. 2011). Furthermore, this PTH-mediated intracellular calcium overloading is proposed as the most likely mechanism that leads to increased oxidative stress in chronic aldosteronism, a condition characterized with elevated PTH levels (Rutledge et al. 2012).

Furthermore, PHPT has been associated with increased risk factors for atherosclerosis, and markers of inflammation. In a study conducted in patients with PHPT and age- and gender-matched controls reported that plasma CRP levels in patients with PHPT were significantly higher than those in the controls ($p = 0.002$), and correlated with systolic and diastolic blood pressure levels (both $r = 0.33$, $p < 0.05$). In addition, plasma TNF-α was significantly elevated in PHPT patients compared with the control group ($p < 0.01$) (Øgard et al. 2005). Moreover, another study reported up-regulation of expression of the gene responsible for inflammation and metabolic functions in PHPT including genes responsible for matrix metalloproteinase (MMP9), chemokine ligand 8 and 13 (CCL8 and 13), tyrosine kinase binding protein (TYROBP), and thrombospondin 1 (TSP 1) (Christensen et al. 2011).

1.3.3 Effects of parathyroid hormone on glucose and insulin metabolism

Elevated PTH levels have also been shown to have effects on insulin and glucose metabolism. A recent study carried out in normotensive, healthy subjects with normal glucose tolerance, plasma PTH level was inversely correlated with insulin sensitivity index (high PTH level is associated with reduced insulin sensitivity). Furthermore, this study reported that plasma PTH is a determinant of the insulin sensitivity index independent from age, gender, waist-to-hip ratio and ethnicity (Chiu et al. 2000). In patients with chronic renal failure (CRF), elevated PTH interferes with the ability of β-cells to augment insulin secretion in response to the insulin-resistant state (Akmal et al. 1985). However, a limited amount of research has been conducted to investigate the cumulative effect of vitamin D and PTH on glucose intolerance, insulin resistance and metabolic syndrome. The data available on this association is not consistent. A community-based study reported that increasing PTH levels are a predictor of increasing prevalence of metabolic syndrome in older men, independent of $25(OH)D_3$, age, season, diabetes and renal function (Reis et al. 2007). However, some studies reported the ability of the active vitamin D metabolite, $25(OH)D_3$, to improve insulin resistance without significant changes in serum calcium or parathyroid hormone levels in

animal models and patients with the renal failure (Mak & Wong 1992). Taken together, it remains unclear whether the effects of PTH are independent of vitamin D.

1.4 Aims and objectives of the thesis

Vitamin D deficiency and secondary hyperparathyroidism, either in combination or independently, have been associated with increased cardiovascular (CV) events with unknown mechanisms. Our group has previously demonstrated that, in an ageing population, low 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentrations are associated with increased concentrations of asymmetric dimethylarginine (ADMA), an eNOS inhibitor and biomarker of increased CV morbidity and mortality. We have not yet demonstrated the relationship in a high risk cohort, such as those with obesity and diabetes, who are at significantly increased risk of CV events, with high prevalence of vitamin D deficiency. Furthermore, we have not yet demonstrated whether the relationship between ADMA and vitamin D is independent of PTH. More importantly, in this study we sought to determine whether vitamin D supplementation will have effects on ADMA, and other markers of nitric oxide signalling, such as TSP-1 and platelet NO responsiveness.

Therefore, the specific aims of this thesis are:

I. To determine whether a low $25(OH)D_3$ level is a predictor of a high ADMA level in patients with obesity and diabetes. We also want to see whether the relationship between vitamin D and ADMA is independent of PTH, renal function and other CV risk factors.

II. To determine whether supplementation with vitamin D_3 will affect;
    a. ADMA concentrations,
    b. hs-CRP levels of healthy subjects without pre-existing CV disease or diabetes.

III. To determine whether supplementation with vitamin D_3 will affect other markers of nitric oxide signalling, specifically TSP-1 and platelet responsiveness to nitric oxide.

Chapter 2 Evaluating the prevalence of vitamin D deficiency and relationship between low vitamin D and elevated parathyroid hormone on markers of endothelial function and inflammation in obese-diabetic patients

2.1 Abstract

Background: Low vitamin D levels and elevated parathyroid hormone (PTH) levels, either in combination or independently, have been associated with increased cardiovascular events. Effects of low vitamin D status and elevated PTH levels on endothelial function, insulin resistance, inflammation and oxidative stress have been documented as possible causes for this association. Asymmetric dimethylarginine (ADMA), a physiologically occurring competitive inhibitor of endothelial nitric oxide producing enzyme, endothelial nitric oxide synthase (eNOS), is a strong marker and mediator of endothelial dysfunction. Furthermore, small elevations of plasma ADMA levels are associated with marked increases in cardiovascular (CV) risk. Previously, our group has demonstrated that low 25-hydroxyvitamin D_3 ($25(OH)D_3$) levels were associated with high concentrations of ADMA and high sensitive C-reactive protein (hs-CRP, a marker of inflammation) in an ageing population. Obesity and Type 2 diabetes mellitus are associated with increased prevalence of vitamin D deficiency and associated with increased risk of adverse cardiovascular events. Furthermore, Type 2 diabetes mellitus & obesity are associated with elevated levels of ADMA. Therefore, the current study evaluated the following:

a) Whether low vitamin D levels are a predictor of high ADMA concentrations;
b) Whether the relationship between vitamin D levels and ADMA concentrations is independent of PTH, renal function or other CV risk factors in obese diabetic patients.

Method: Plasma concentrations of $25(OH)D_3$ (marker of vitamin D status), ADMA, PTH, hs-CRP, HbA_1C (marker of glycemic control), renal function and liver function and other biochemical parameters were assessed in an obese (body mass index (BMI) 36.9±6.8 (30.21-64.71) kg/m^2) diabetic cohort (n=164) aged 35-88 years (mean±SD 64.8±11.2 years). In all cases, a detailed medical history was obtained at entry with emphasis on blood pressure, current medication, and CV risk factors. No patient was on vitamin D supplementation at the

time of study entry. Patients with the following conditions were excluded: treatment initiation with angiotensin-converting enzyme (ACE) inhibitors or statins in the previous 3 months, severe renal impairment (calculated creatinine clearance < 30 mL/min/1.73 m^2) and pregnancy and lactating.

Results: Mean 25(OH)D_3 and median PTH levels were 60.59±24.25 nmol/L and 4.55 (0.90-20.40) pmol/L respectively. There were 38% of patients with vitamin D deficiency (25(OH)D_3 < 50 nmol/L), and 40% with PTH levels above 5.8 pmol/L. Based on univariate analyses, high ADMA concentrations were positively associated with PTH level (R=0.23, p<0.01), whereas 25(OH)D_3 (R=0.06, p=0.46) and hs-CRP levels (R=-0.06, p=0.47) were not. While there was a direct relationship between HbA_1C and ADMA (R=0.18, p<0.05); CrCL (R=-0.12, p=0.19), calcium (R=-0.07, p=0.40), and phosphate level (R=0.02, p=0.80) were not correlates of ADMA. Multiple linear regression analyses corrected for age, gender, blood collected season, BMI, 25(OH)D_3 level, creatinine clearance (CrCL), hs-CRP, statins, angiotensin converting enzyme (ACE) inhibitors or metformin use, and CV risk factors, showed that the independent correlates of ADMA concentration were: PTH (β=0.28, p<0.01) and HbA_1C level (β=0.25, p<0.01). Liver and renal functions were well preserved in all subjects.

Conclusion: While ADMA concentration in the general population varies directly with CRP and inversely with vitamin D concentrations, in obese diabetics these relationships are not apparent: instead, PTH and HbA1C emerge as the major correlates of elevated ADMA concentrations independent from vitamin D status and other CV risk factors. The basis for this interaction, its implications regarding redox stress/endothelial dysfunction in obese diabetes and its potential therapeutic utility are worthy of further examination.

2.2 Introduction

The "classical" effects of vitamin D and parathyroid hormone (PTH) are to regulate calcium and mineral homeostasis (Potts Jr & Deftos 1969; Sahota et al. 2004). Recently, in the last 5 years, it is increasingly suggested that vitamin D is important in cardiovascular homeostasis (Lee, J.H. et al. 2008; Temmerman 2011; Wang et al. 2012; Wang, T.J. et al. 2008), and conversely, that vitamin D deficiency may represent a risk factor for the development of coronary disease (Szeto et al. 2012; Zittermann, A. & Koerfer 2008). Low vitamin D levels lead to reduced intestinal absorption of calcium and thus, triggers the secretion of PTH from parathyroid glands (Canaff & Hendy 2002; Rasmussen 1961), which in turn is responsible for increasing calcium levels by: 1) mobilizing calcium from bone, 2) enhancing intestinal absorption of calcium, and 3) reducing calcium loss in urine (Fleet et al. 1994; Gensure, Gardella & Jüppner 2005; Rasmussen 1961). In addition to its effects in regulating calcium and mineral homeostasis, elevated levels of PTH with either primary hyperparathyroidism or secondary hyperparathyroidism, have also been associated with increased adverse cardiovascular outcomes (Block et al. 2004; Hagström et al. 2009; Palmer et al. 2011; Rostand & Drüeke 1999; Slinin, Foley & Collins 2005; Yu et al. 2010). Patients with chronic elevation of PTH levels are at increased risk of insulin resistance (Akmal et al. 1985; Chiu, K.C. et al. 2004), dyslipidemia (Lim et al. 1998; Zanos, Mitsopoulos & Sakellariou 2005), heart failure (Bogin, Massry & Harary 1981; Rostand & Drüeke 1999), hypertension (Hulter et al. 1986; Snijder et al. 2007), cardiac hypertrophy (Saleh et al. 2003; Symons et al. 1985), and myocardial infarction (Anderson, Jeffrey L et al. 2011).

Impaired vascular endothelial function has been suggested as a possible mechanism to explain the nexus between vitamin D deficiency and increased adverse cardiovascular outcomes (Sugden et al. 2008; Tarcin et al. 2009). In addition, elevated PTH has also been found to be associated with increased arterial stiffness and impaired endothelial function, which is improved after lowering PTH with parathyroidectomy (Kosch et al. 2000; Schillaci, Giuseppe et al. 2011). Previously, we have shown that in an ageing population, low vitamin D levels are associated with elevated plasma concentrations of asymmetric dimethylarginine (ADMA) (Ngo, D.T. et al. 2010). ADMA is an endogenous inhibitor of endothelial nitric oxide synthase (eNOS) (Miyazaki et al. 1999), has been shown to be a critical circulating biomarker of endothelial function, and adverse cardiovascular outcomes (Valkonen et al. 2001). A small increase in ADMA is associated with marked increase in cardiovascular mortality (Schnabel et al. 2005; Valkonen et al. 2001). However, we did not assess the

relationship between ADMA and PTH in this aging population (Ngo, D.T. et al. 2010). Given the high prevalence of low vitamin D and high parathyroid hormone (PTH) levels in obese diabetic patients, we sought to examine whether there is a relationship between ADMA and vitamin D and whether this relationship is independent of PTH levels in obese diabetic patients.

2.3 Aims and hypotheses of the study

This study investigated:

I. whether the low 25(OH)D_3 level is a predictor of a high ADMA level in this high risk obese diabetic patients.
II. whether the relationship between 25(OH)D_3 and ADMA concentrations is independent of PTH, renal function or other CV risk factors of these obese diabetics.

Therefore, the hypotheses examined were:

I. low 25(OH)D_3 level is a predictor of high ADMA levels in obese diabetic subjects.
II. the relationship between 25(OH)D_3 and ADMA concentrations is independent of PTH, renal function, or other CV risk factors of these obese diabetics.

2.4 Study protocol

All of the work including interviewing the study subjects and their recruitment, measurement of the biological marker ADMA, data collection, data entry and maintenance of database and statistical data analysis was performed by myself.

2.4.1 Study subjects

In this study we selected a cohort of obese-diabetic patients who were free-living, free from kidney and liver dysfunctions and not on vitamin D supplements at the time of recruitment. There were several reasons for selecting this patient cohort:

a. Obesity and type 2 diabetes mellitus have associated with an increased prevalence of vitamin D deficiency (Caballero 2003). It has been suggested that obese patients (BMI $\geq$ 30 Kg/m^2) are more likely to require a larger than usual dose of vitamin D_3 (Gallagher, Yalamanchili & Smith 2013). Vitamin D is a fat soluble vitamin and is readily sequestered into fat stores (Blum et al. 2008). It has been found that when given the same dose of vitamin D (i.e., 50,000 IU) obese patients had a significantly smaller increase in plasma vitamin D concentration compared to non-obese patients (Wortsman et al. 2000). Furthermore, the prevalence of vitamin D deficiency ($25(OH)D_3$ level < 50 nmol/L) in morbidly obese patients was reported to be as high as 61% and 90% for combined vitamin D deficiency and insufficiency ($25(OH)D_3$ level < 75 nmol/L) (Goldner et al. 2008). Equally, patients with Type 2 diabetes mellitus have been found to have an increased prevalence of vitamin D deficiency (Muscogiuri et al. 2012). More recently, number of observational studies reported the possible association between lower vitamin D status and higher risk of incident type 2 diabetes (Knekt et al. 2008; Mitri, Muraru & Pittas 2011; Pittas, A.G. et al. 2007).

b. Obesity and diabetes mellitus are risk factors for adverse cardiovascular events (Braunwald 1997). Obesity is frequently associated with development of insulin-resistance, which is in turn associated with endothelial dysfunction (Braunwald 1997; Hossain, Kawar & El Nahas 2007; Reaven 1993).

The criteria used for vitamin D deficiency was $25(OH)D_3$ < 50 nmol/L. Consecutive patients with diabetes and obesity (n=164) (BMI) $\geq$ 30 Kg/m^2) were recruited at out-patient cardiology clinics of The Queen Elizabeth Hospital (TQEH), South Australia, where 67% of the patients were recruited during the spring and summer and the rest (33%) were recruited during the autumn and winter. Patients were enrolled in this study if they were diagnosed for type 2 diabetes mellitus, were obese (body mass index (BMI) $\geq$ 30 Kg/m^2), ambulant and able to give informed consent. Patients with severe renal impairment (calculated creatinine clearance < 30 ml/min/1.73 m^2), or those on pre-existing vitamin D therapy were excluded from the study. Patients were also excluded if they had the initiation of treatment with angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARB) or statins in the previous 3 months or were pregnant/lactating. In all cases, a detailed medical history was obtained at entry with an emphasis on blood pressure, current medication, CV

risk factors and any current vitamin D supplementation. Height, weight, BMI and the season blood was collected in were recorded for all subjects and blood was taken via an antecubital vein for the determination of routine biochemical parameters. All volunteers gave informed consent before the study. The study was approved by the Human Research Ethics Committees of TQEH and the University of South Australia.

2.4.2 Biochemical parameters tested:

- Vitamin D (25-hydroxyvitamin D_3 [$25(OH)D_3$])
- Parathyroid hormone (PTH)
- Ionised and total calcium, phosphate
- Serum creatinine concentrations
- Fasting glucose levels
- Glycated hemoglobin (HbA_1C)
- High sensitive C-reactive protein (hs- CRP)
- Plasma ADMA, SDMA and arginine concentrations
- Total protein levels and liver function
- Lipid profiles

a. $25(OH)D_3$ levels were measured by a commercially available enzyme immunoassay (Biokit, Barcelona, Spain)/competitive binding chemiluminescence assay after extraction (Immunodiagnostic Systems, Boldon, UK). This assays has a 100% specificity for detecting $25(OH)D_3$ levels. The assays were carried out by SA Pathology, Frome road, Adelaide, South Australia and the details of the methods are attached (Appendix B).

b. PTH (intact PTH) levels were measured by commercially available electrochemi luminescence immunoassay method (Roche Diagnostics, Indianapolis, IN). The assay was carried out by SA Pathology, Frome road, Adelaide, South Australia and the detailed method is attached (Appendix C).

c. High-sensitive C-reactive protein (hs-C-reactive protein) concentrations, as a marker of systemic inflammatory activation, lipid profile, creatinine, total serum calcium and

ionised calcium were measured by a 1251 radio-immunoassay (Immunodiagnostic Systems Ltd, Bolden, UK). The assays were carried out by SA Pathology, Frome road, Adelaide, South Australia.

d. Renal function was assessed by creatinine clearance by SA Pathology, Frome road, Adelaide, South Australia. The creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault equation (Cockcroft & Gault 1976) and indexed for body surface area with the Dubois and Dubois formula (DuBois & DuBois 1915).

e. Liver function tests were carried out to evaluate liver dysfunction (bilirubin, aminotransferases, and alkaline phosphatase levels were measured) by SA Pathology, Frome road, Adelaide, South Australia.

f. Asymmetric dimethylarginine (ADMA) levels were measured by high performance liquid chromatography (HPLC) method. Measurements of all plasma concentrations of ADMA were carried-out by myself at the Cardiology Research Laboratory, Basil Hetzel Institute for Translational Health Research, Adelaide, South Australia.

2.4.2.1 Asymmetric dimethylarginine assay (Quantification of asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and arginine)

In all patients, blood was collected into heparinised blood collection tubes and centrifuged at 4ºC at 2700 x g for 20 minutes (min) and supernatant plasma was stored at -80 ºC until assayed. Concentrations of these methylated arginine residues in plasma were measured by HPLC using derivatization with a fluorescent active agent after solid-phase extraction.

The method used in our laboratory to assess arginine residues has been described in detail previously (Heresztyn, Worthley & Horowitz 2004). Herewith, this method is included briefly, highlighting changes from the previously described method.

Sample preparation and solid-phase extraction

The two derivatives of arginine (ADMA, SDMA) and arginine itself were determined using 150 µL of the centrifuged plasma combined with 60 µL of internal standard L-NMMA (20 µg/mL)

and 300 µL of 10% 5-sulfosalysilic acid and made up to 1.9 mL with purified water. The prepared plasma solutions were vortexed and kept on ice for 10 min to allow complete protein precipitation and then centrifuged at 9000g for 2 min. Solid-phase extraction was carried out with a Gilson GX-274 automated liquid extractor. The supernatant was loaded on to Bond Elut SCX 100mg cartridges conditioned with 1 mL methanol and followed by 2 mL of freshly prepared 2% (w/v) trichloroacetic acid. The plasma solutions were passed through the BOND ELUT-SCX (100 mg 1 Ml; Lot number 1706003) cartridges (VARIAN, Agilent Technologies, USA) at about 3-4 mL per minute and unwanted plasma components rinsed off with 3 ml of 0.1M sodium phosphate buffer (pH 6) followed by 1 mL methanol. ADMA/SDMA and arginine were eluted from the solid phase with 2 ml freshly prepared 2% triethylamine in 65% methanol/water. The eluents were dried under a stream of nitrogen at 55 ºC. The residues were re-dissolved in 150 µL of purified water and centrifuged at 9000g for 2 min to remove particulates prior to derivatization with fluorescent active agent.

Sample derivatization

Reconstituted plasma extracts (50 µL) and 50 µL of aqueous borate buffer (0.2M, pH 8.8) were combined with 10 µL of fluorescent derivatizing reagent 10 mM (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) in acetonitrile in low volume HPLC vial inserts under vigorous vortexing for several seconds. According to the previous data (Heresztyn, Worthley & Horowitz 2004), the reaction was completed after 1 min and the resulting derivatives were stable for at least 1 week at room temperature.

Measurement with high-performance liquid chromatography

The fluorescent derivatives were injected to 5 µ Luna C18 column with guard column (C18 cartridge) at 40 ºC for ADMA/SDMA and 30 ºC for arginine with an acetonitrile gradient in 0.1M sodium acetate buffer at a flow rate of 1mL per minute.

Two different mobile phase compositions were used to generate this gradient. Mobile phase A solution was 4% acetonitrile in 0.1M sodium acetate (pH 6) and mobile phase B solution was 30% acetonitrile in 0.1M sodium acetate (pH 6).

The mobile phase gradient conditions for separation of ADMA/SDMA were A:B = 96.3:3.7 (19 min), then 84.5:15.5 over 0.4 min, hold until 29.5 min, and flushed with 100% B until 33 min followed by return to the initial conditions 96.3:3.7 at 33 min allowing the column to re-equilibriate for 8 min. Conditions were maintained by two "Waters 510 pumps with an automated gradient controller. The ADMA/SDMA analyses used 20 µL injections of the fluorescent-derivatized samples.

The mobile phase gradient conditions for the separation of arginine were A:B = 96:4 (20 min), then 84:16 over 0.4 min, hold until 29.5 min then flushed with 100% B until 33 min followed by a return to initial conditions 96:4 at 33 min, while allowing the column to re-equilibriate for 8 min. The arginine analyses used 2.5 µL injections of the fluorescent-derivatized samples.

Analytes were detected by a "Perkin-Elmer (Melbourne, Australia) LS40" fluorescence detector at an excitation wavelength of 250 nm and emission wavelength of 395 nm. All data were collected with "Laura LabLogic (version 1.2) HPLC software" for windows (Daintree Scientific, St. Helens, Tasmania, Australia).

Standard curves and quality control of the assay

Calibration standards for ADMA and SDMA (0.05, 0.25, 0.5, 0.7, 1 µg/mL) and arginine (5, 10, 15, 20, 30 µg/ml) were prepared from stock solutions (0.4 mg/ml in 0.1M HCl) diluted in water and stored at −20 ◦C.

Standard curves for ADMA/SDMA and arginine were prepared by combining 150 µL of the standard solution and 60 µL of internal standard with 150 µL of plasma (pooled plasma from five healthy volunteers) and extracting, derivatizing and analysing using the procedures detailed above in the sections. Calibration curves were calculated by graphing the ratio of peak area (arginine, ADMA or SDMA) to area of the internal standard (peak area ratio) versus concentration. Analyte recovery for accuracy and precision was determined by spiking plasma from healthy volunteers with known quantities of arginine and dimethylarginines (ADMA/SDMA), extracting and quantitating spiked components from the standard curve (quality control (QC) samples). Medium and high QC samples of ADMA/SDMA were prepared by spiking with 0.05 µg/ml (0.25 µmol/L) and 0.2 µg/ml (1 µmol/L) of extra quantity of the compound respectively. Medium and high QC samples of arginine were

prepared by spiking with 5 µg/ml (29 µmol/L) and 10 µg/ml (57 µmol/L) of extra quantity of arginine, respectively.

Reproducibility of the assay and recovery rate of arginine residues

Intra-assay/inter-assay variability and recoveries of the validated assay (Heresztyn, Worthley & Horowitz 2004) of ADMA/SDMA and arginine residues in our laboratory are given in Table 2.1. Inter-assay variability of arginine residues were calculated by data collected from five consecutive assays done at the same time period where the patient samples were performed. Percentage recoveries were calculated by the amount of compound recovered relative to the extra quantities of the compounds added to the medium and high QC samples.

Table 2.1 Reproducibility of ADMA assay and the recovery rate

Compound	**Intra-assay variability (%) (n=5)**	**Inter-assay variability (%) (concentration range µmol/L) (n=5)**	**Recovery rate (%)**
ADMA	≤ 2.3	2.6-6.2 (0.494-1.383)	92-97
SDMA	≤ 5	4.2-6.9 (0.445-1.334)	93-94
Arginine	≤ 2	5.4-7.0 (119.97-163.59)	81-85

n = number of runs within the assay/number of runs in between the assay.

The ADMA assay had a a coefficient of variance (CV) of less than 2.5% within run and CV of less than 6.5% in between the runs. The recovery rate for ADMA was 92-97% and the detection limit of the assay was 0.1 µmol/L.

2.4.3 Statistical analyses

All data are expressed as mean ± SD unless otherwise stated. Normal distribution was tested for all continuous variables, and skewed data were normalized by either log or square root transformation. All analyses were performed with SPSS version 21.0 software (IBM SPSS Statistics, Chicago, IL, USA), and a *p*-value of < 0.05 was considered to be statistically significant. All graphs/figures were generated with GraphPad Prism version 6 software (GraphPad Software, Inc., San Diego, California, USA).

Correlations of vitamin D (25-(OH)D_3) levels or PTH levels with other cardiovascular risk parameters were analysed by linear regression analyses. Multivariate analysis (backward

multiple linear regression analysis) was utilized to identify independent determinants of ADMA levels in these obese diabetic cohort. Variables included to predict elevated ADMA levels were age, gender, BMI, corrected CrCL, PTH, vitamin D (25-(OH)D_3), HbA_1C, hs-CRP, statins or ACEI/ARB or metformin use and CV risk factors including hypertension and hypercholesterolemia.

2.5 Results

2.5.1 Patient characteristics

The patients' baseline clinical characteristics are summarized in Table 2.2. These diabetic patients were obese with an average BMI of 36.9±6.8 Kg/m^2. In general, renal function was well preserved with mean creatinine clearance of 80.7±28.5 mL/min/1.73 m^2.

According to the definition of vitamin D deficiency (Section 1.2.2), 61 subjects (38%: data for 160 subjects were available), were vitamin D deficient (25-(OH)D_3 < 50 nmol / L and further 57 patients (36%) were vitamin D insufficient (i.e., 25-(OH)D_3 50-75 nmol/L). Therefore, the total prevalence of vitamin D inadequacy (25-(OH)$D_3 \leq$ 75 nmol/L) was 74% in these patients. There were 40% of subjects with PTH levels above the reference limit (5.8 pmol/L) with a median PTH level of 4.55 (0.90-20.40) pmol/L. Mean plasma ADMA concentrations were slightly elevated from the previously reported normal range (mean value for the healthy population = 0.4 to 0.6 µmol/L)(Horowitz & Heresztyn 2007) for the method used in our laboratory. Ninety-one patients (61%) had elevated ADMA levels from the normal range, ranging from 0.60-0.98 µmol/L. The inflammatory status measured by hs-CRP was significantly higher in this population with measured levels (median 4.10 [0.38-100] mg/L) in 95 subjects (out of 156 for whom data was available: 61%) higher than 3 mg/L.

The mean levels of ionised calcium (1.10-1.30 mmol/L), total calcium (2.10-2.55 mmol/L), phosphate (0.65-1.45 mmol/L) and total cholesterol (1.5-9.2 mmol/L) were within the respective normal ranges. Mean plasma PTH levels were slightly elevated from the normal range (0.8-5.5 pmol/L). Sixty three (40%) obese diabetic patient's PTH levels were elevated from the normal reference range with a mean of 8.7 pmol/L. Both glycosylated hemoglobin percentage and random glucose levels were higher in this obese diabetic cohort with a mean random glucose level of 9.28±4.55 mmol/L (range 2.3-31.0 mmol/L). In general, renal function was well preserved with mean of creatinine clearance 80.7±28.5 mL/min/1.73 m^2.

There was no patient with adjusted creatinine clearance less than 30 mL/min/1.73 m^2. Clinically significant hepatic dysfunction was not recorded for any of the patients. The biochemical findings are described in Table 2.3.

Table 2.2 Patient characteristics of obese-diabetic cohort (n=164)

Parameter	N	%
Age (years) (mean±SD)	64.8±11.2 (35-88)^	
BMI (Kg/m^2)	36.9±6.8 (30.21-64.71)^	
Gender (% male)	97/164	59.1
Previous angina/MI	78/161*	48.4
History of hypercholesterolemia	124/163*	76.1
Hypertension	111/162*	68.5
Atrial fibrillation	27/164	16.5
History of heart failure	26/164	15.8
Statin therapy	98/164	59.8
ACEI/ARB therapy	96/164	58.5
Antiplatelet drugs	61/164	37.2
Metformin treatment	88/164	53.7

ACEI = angiotensine converting enzyme inhibitors; ARB = angiotensine receptor blockers; BMI = body mass index; MI = myocardialo infarction; N = value or number of patients; % = percentage of subjects out of total.

* Indicates the number of subjects for whom the data are available

^ Mean±SD (minimum-maximum)

Table 2.3 Biochemical data of obese-diabetic patients (n=164)

	Mean	**SD**	**Minimum**	**Maximum**
Vitamin D (25-(OH)D_3) (nmol/L)	60.6	24.2	18	130
PTH (pmol/L)	5.6	3.4	0.9	20.4
hsCRP (mg/L)	7.2	10.5	0.38	100
HbA_1C (%)	7.9	1.7	5.3	13.6
CrCL (indexed for BSA) (mL/min/1.73 m^2)	80.7	28.5	36.4	176.3
Ionised calcium (mmol/L)	1.2	0.05	1.1	1.4
Total calcium (mmol/L)	2.4	0.1	2.1	2.8
Phosphate (mmol/L)	1.1	0.2	0.7	1.7
Calcium phosphate product $(mmol/L)^2$	2.7	0.7	4.22	1.81
Total cholesterol (mmol/L)	4.2	1.2	1.5	9.2
ADMA (µmol/L)	0.63	0.11	0.4	0.98
SDMA (µmol/L)	0.61	0.18	0.31	1.17
Arginine (µmol/L)	107.46	32.98	44.76	213.86
Random glucose (mmol/L)	9.28	4.55	2.30	31.00
Total protein (g/L)	74.44	5.01	59.0	87.0

ADMA = asymmetric dimethyl arginine; BSA = body surface area; CrCL = creatinine clearance; HbA_1C = glycated hemoglobin; hs-CRP = hs-C-reactive protein; 25(OH)D_3 = 25-hydroxy vitamin D_3; PTH = parathyroid hormone; SDMA = symmetric dimethyl arginine; SD = standard deviation.

2.5.2 Univariate analyses

2.5.2.1 Correlates of vitamin D_3 ($25(OH)D_3$) levels

Univariate analyses between $25(OH)D_3$ levels and the various continuous and categorical variables are contained in Table 2.4.

Significant correlations of $25(OH)D_3$ levels with continuous variables (PTH, ionized calcium and age) are illustrated in Figure 2.2. Glycosylated hemoglobin (HbA_1C), random glucose levels, creatinine clearance, phosphate and calcium-phosphate product or total cholesterol were not significant correlates of $25(OH)D_3$ levels. Age and ionized calcium levels in the blood were positively associated with $25(OH)D_3$ levels. A borderline significant correlation was found between both BMI and total calcium levels when compared with with vitamin D levels ($25(OH)D_3$). A significant inverse correlation was shown between $25(OH)D_3$ levels and PTH concentrations. Furthermore, $25(OH)D_3$ levels were positively correlated with age and ionised calcium levels (Figure 2.2). The presence of high cholesterol levels was associated with high plasma $25(OH)D_3$ levels, however, other risk factors including hypertension, and atrial fibrillation were not. Treatment with statin or ACEI/ARB was also associated with high vitamin D ($25(OH)D_3$) concentrations.

Table 2.4 Univariate correlates of $25(OH)D_3$ levels (n=164)

(A) Continuous univariate correlates of $25(OH)D_3$ levels of obese-diabetic patients

Parameter	**Standardized coefficient (β)**	**Significance (p)**
Age (years)	0.167	0.03*
BMI (Kg/m^2)	-0.172	0.051
PTH (pmol/L)	-0.264	0.001*
hsCRP (mg/L)	-0.110	0.17
HbA_1C (%)	-0.100	0.21
Random glucose (mmol/L)	-0.003	0.98

Parameter	Standardized coefficient (β)	Significance (p)
CrCL (indexed for BSA) (mL/min/1.73 m^2)	-0.016	0.86
Ionised calcium (mmol/L)	0.182	0.02*
Total calcium (mmol/L)	0.148	0.06
Phosphate (mmol/L)	0.070	0.38
Calcium phosphate product $(mmol/L)^2$	0.036	0.65
Total cholesterol (mmol/L)	-0.095	0.24
ADMA (µmol/L)	0.061	0.46
SDMA (µmol/L)	0.003	0.974
Arginine (µmol/L)	0.014	0.866

ADMA = asymmetric dimethyl arginine; BMI = body mass index; BSA = body surface area; CrCL = creatinine clearance; HbA_1C = glycated hemoglobin; hs-CRP = hs-C-reactive protein; $25(OH)D_3$ = 25-hydroxy vitamin D_3; PTH = parathyroid hormone; SDMA = symmetric dimethyl arginine. * $p<0.05$, ** $p<0.001$

(B) Categorical univariate correlates of $25(OH)D_3$ levels of obese-diabetic patients

Differences in mean levels were performed using the independent sample t-test.

	Mean±SD of $25(OH)D_3$ levels (nmol/L) Yes Vs No	**Significance (p value)**
Gender (male Vs female)	62.6±25.0 Vs 57.6±22.9	0.20
Hypertension	62.4±23.5 Vs 57.4±25.8	0.23
Hypercholesterolemia	62.5±25.4 Vs 54.4±19.7	0.04*
History of ischemia	61.2±25.5 Vs 60.5±23.4	0.86

	Mean±SD of 25(OH)D_3 levels (nmol/L) Yes Vs No	**Significance (p value)**
History of heart failure	67.2±26.8 Vs 59.3±23.6	0.13
Atrial fibrillation	66.6±28.8 Vs 59.4±23.2	0.17
Metformin treatment	61.9±24.0 Vs 59.1±24.6	0.47
Statin therapy	64.3±25.9 Vs 55.1±20.6	0.02*
Use of ACEI/ARB	64.1±24.5 Vs 55.7±23.2	0.03*

ACEI = angiotensine converting enzyme inhibitors; ARB = angiotensine receptor blockers; SD = standard deviation.

* $p<0.05$

In these obese diabetic patients, 25(OH)D_3 levels were not correlated with either hs-CRP or ADMA concentrations, as expected (Figure 2.1).

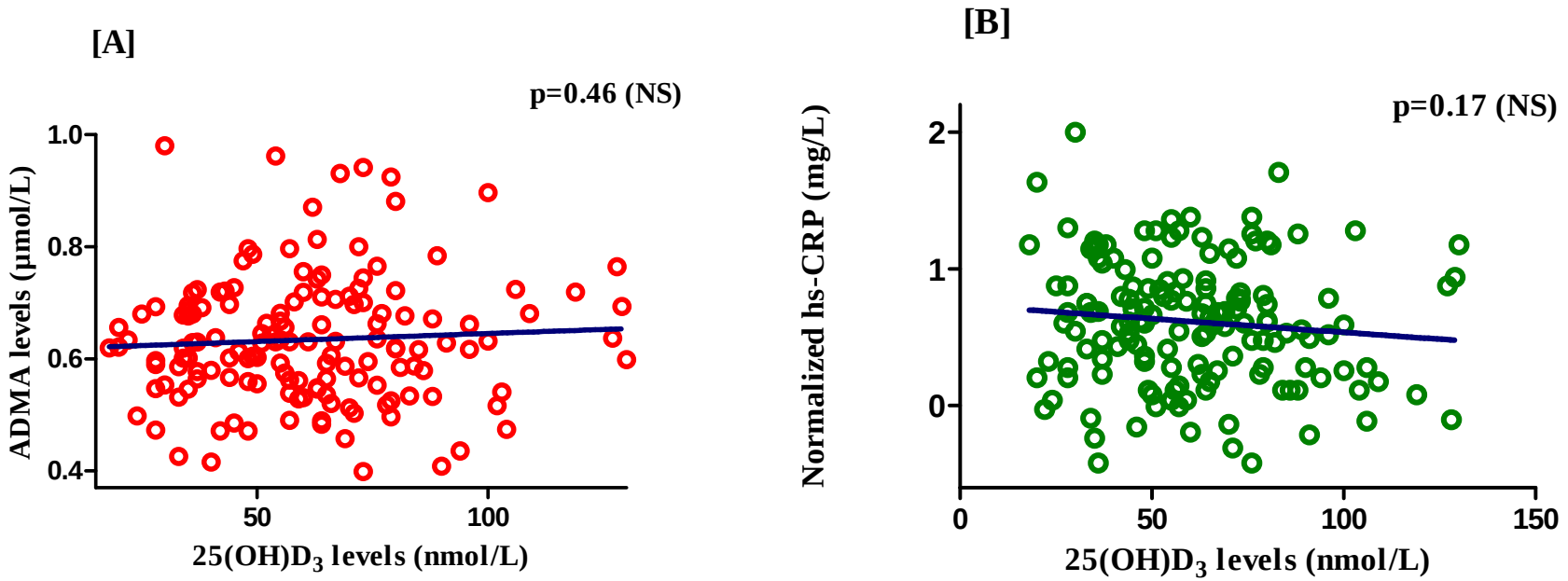

Figure 2.1 Baseline vitamin D_3 ($25(OH)D_3$) levels vs [A] baseline ADMA concentrations ($\beta = 0.061$, $p = 0.46$); [B] baseline hs-CRP levels ($\beta = -0.110$, $p = 0.17$) of the obese diabetics.

NS = not significant

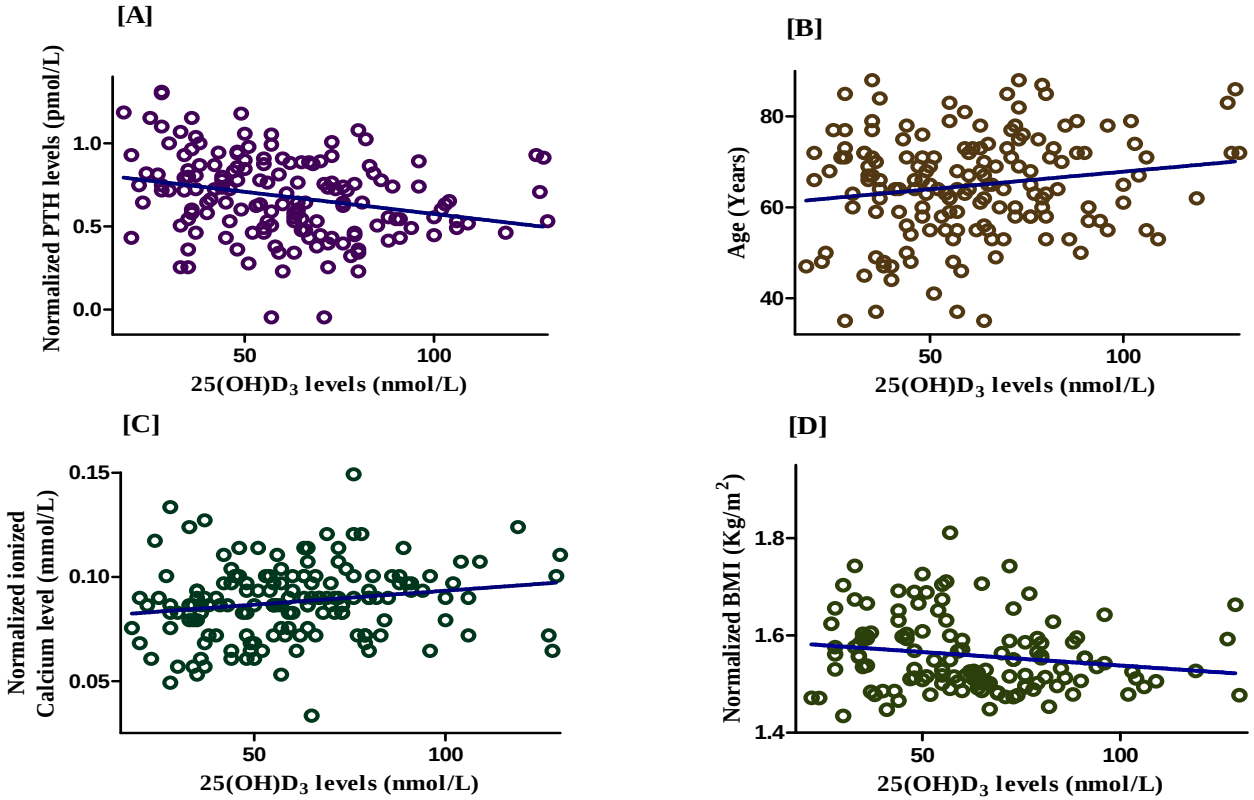

Figure 2.2 Correlation of 25(OH)D_3 levels with [A] PTH levels (there was an inverse correlation with 25(OH)D_3 levels: $\beta = -0.26$, $p = 0.001$) and [B] Age (there was a positive correlation with 25(OH)D_3 levels: $\beta = 0.167$, $p = 0.03$); [C] Ionized calcium levels (there was a positive correlation with 25(OH)D_3 levels: $\beta = 0.182$, $p = 0.02$); [D] BMI (there was an borderline inverse correlation with 25(OH)D_3 levels: $\beta = -0.17$, $p = 0.051$) of the obese diabetics.

25(OH)D_3 = 25-hydroxyvitamin D_3; PTH = parathyroid hormone; BMI = body mass index.

2.5.2.2 Correlates of parathyroid hormone (PTH) levels

Univariate analyses were carried out to identify the effects of plasma intact PTH levels with cardiovascular risk factors in these obese diabetics (Table 2.5). Correlations of PTH level with the continuous variables are summarized in Figure 2.3. A borderline positive correlation was seen between BMI/age with PTH levels. 25(OH)D_3, creatinine clearance, ionized calcium and total calcium levels were negatively correlated with intact PTH (Figure 2.3). However, the association between PTH and hs-CRP levels was not significant. A significant relationship was seen between PTH levels and ADMA/SDMA concentration in obese diabetics without any renal insufficiency (Figure 2.3). The use of anti-diabetic treatment with metformin was associated with low PTH levels. Diabetic patients with atrial fibrillation had significantly higher PTH levels when compared to patients without atrial fibrillation.

Table 2.5 Univariate correlates of PTH levels (n=164)

(A) Continuous univariate correlates of PTH levels of obese-diabetic patients

Parameter	**Standardized coefficient (β)**	**Significance (p)**
Age (years)	0.156	0.050
BMI (Kg/m^2)	0.165	0.06
$25(OH)D_3$ (nmol/L)	-0.264	0.001*
hsCRP (mg/L)	0.134	0.10
HbA_1C (%)	-0.039	0.63
Random glucose (mmol/L)	-0.167	0.10
CrCL (indexed for BSA) ($mL/min/1.73\ m^2$)	-0.280	0.002*
Ionised calcium (mmol/L)	-0.253	0.001*
Total calcium (mmol/L)	-0.223	0.005*
Phosphate (mmol/L)	-0.045	0.57
Calcium phosphate product $(mmol/L)^2$	-0.084	0.30
Total cholesterol (mmol/L)	0.024	0.76
ADMA (µmol/L)	0.229	0.005*
SDMA (µmol/L)	0.428	0.000**
Arginine (µmol/L)	-0.045	0.583

ADMA = asymmetric dimethyl arginine; BMI = body mass index; BSA = body surface area; CrCL = creatinine clearance; HbA_1C = glycated hemoglobin; hs-CRP = hs-C-reactive protein; $25(OH)D_3$ = 25-hydroxy vitamin D_3; PTH = parathyroid hormone; SDMA = symmetric dimethyl arginine. * $p<0.05$, ** $p<0.001$

(B) Categorical univariate correlates of PTH levels of obese-diabetic patients

Differences in median levels were performed using the *Mann-Whitney U* test.

	Median of PTH levels (pmol/L) Yes Vs No	**Significance (p value)**
Gender (male Vs female)	4.40 Vs 5.30	0.41
Hypertension	5.20 Vs 4.40	0.52
Hypercholesterolemia	5.05 Vs 4.40	0.60
History of ischemia	5.20 Vs 4.40	0.42
History of heart failure	5.75 Vs 4.40	0.20
Atrial fibrillation	5.70 Vs 4.40	0.03*
Metformin treatment	4.00 Vs 5.80	0.000**
Statin therapy	5.20 Vs 4.40	0.23
Use of ACEI/ARB	4.85 Vs 4.40	0.63

ACEI = angiotensine converting enzyme inhibitors; ARB = angiotensine receptor blockers.

* $p<0.05$, ** $p<0.001$

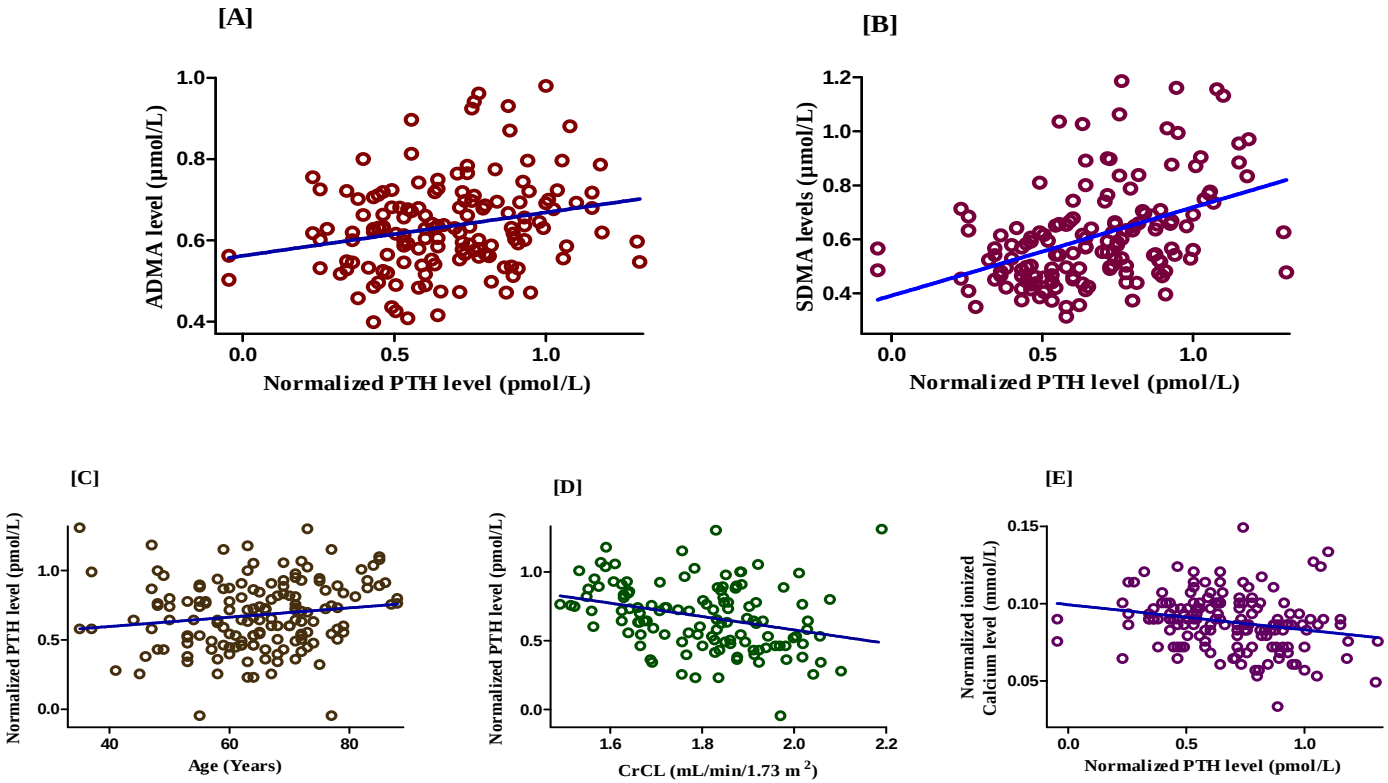

Figure 2.3 Correlation of PTH levels with [A] ADMA concentrations (there was a positive correlation with PTH levels: β = 0.229, p = 0.005); [B] SDMA concentrations (there was a positive correlation with PTH levels: β = 0.428, p<0.001); [C] Age (there was a borderline positive correlation with PTH levels: β = 0.156, p = 0.05); [D] CrCL (there was an inverse correlation with PTH: β = -0.280, p = 0.002); and [E] Ionized calcium levels (there was an inverse correlation with PTH: β = -0.253, p = 0.001) of the obese diabetics.

ADMA = asymmetric dimethyl arginine; PTH = parathyroid hormone; CrCL = creatinine clearance.

2.5.2.3 Correlates of asymmetric dimethylarginine (ADMA) levels

Correlates of ADMA versus continuous and categorical variables are documented in Table 2.6. The univariate analyses showed that PTH (Figure 2.3) and HbA_1C (Figure 2.4) levels have a significant positive correlation with ADMA concentrations. A borderline significant positive correlation was shown between ADMA and BMI (Figure 2.4), but ADMA concentrations were not associated with renal function measured by creatinine clearance (CrCL) (β=-0.12; p=0.19), total calcium (β=-0.07; p=0.40) and phosphate (β=-0.07; p=0.40) levels. Patients with history of heart failure reported higher ADMA values than those without any past heart failure event. Figure 2.4 summarizes the correlation of ADMA levels with HbA_1C and BMI levels.

Table 2.6 Univariate correlates of ADMA levels (n=164)

(A) Continuous univariate correlates of ADMA levels of obese-diabetic patients

Parameter	**Standardized coefficient (β)**	**Significance (p)**
Age (years)	0.129	0.12
BMI (Kg/m^2)	0.167	0.06
$25(OH)D_3$ (nmol/L)	0.061	0.46
PTH (pmol/L)	0.229	0.005*
HbA_1C (%)	0.185	0.025*
Random glucose (mmol/L)	-0.088	0.14
CrCL (indexed for BSA) (mL/min/1.73 m^2)	-0.12	0.19
Total cholesterol (mmol/L)	-0.073	0.38
hsCRP (mg/L)	-0.060	0.47

ADMA = asymmetric dimethyl arginine; BMI = body mass index; HbA_1C = glycated hemoglobin; hs-CRP = hs-C-reactive protein; $25(OH)D_3$ = 25-hydroxy vitamin D_3; PTH = parathyroid hormone. * $p<0.05$.

(B) Categorical univariate correlates of ADMA levels of obese-diabetic patients

Differences in mean levels were performed using the independent sample t-test**.**

	Mean±SD of ADMA (µmol/L) Yes vs No	**Significance (p value)**
Gender (male Vs female)	0.62±0.11 vs 0.65±0.12	0.19
Hypertension	0.64±0.12 vs 0.63±0.10	0.71
Hypercholesterolemia	0.64±0.12 vs 0.63±0.10	0.91
History of ischemia	0.64±0.11 vs 0.63±0.11	0.49
History of heart failure	0.67±0.14 vs 0.63±0.10	0.06
Atrial fibrillation	0.66±0.14 vs 0.63±0.11	0.29
Metformin treatment	0.62±0.12 vs 0.65±0.11	0.13
Statin therapy	0.64±0.12 vs 0.63±0.10	0.80
Use of ACEI/ARB	0.63±0.11 vs 0.64±0.11	0.40

ACEI = angiotensine converting enzyme inhibitors; ARB = angiotensine receptor blockers;

SD = standard deviation.

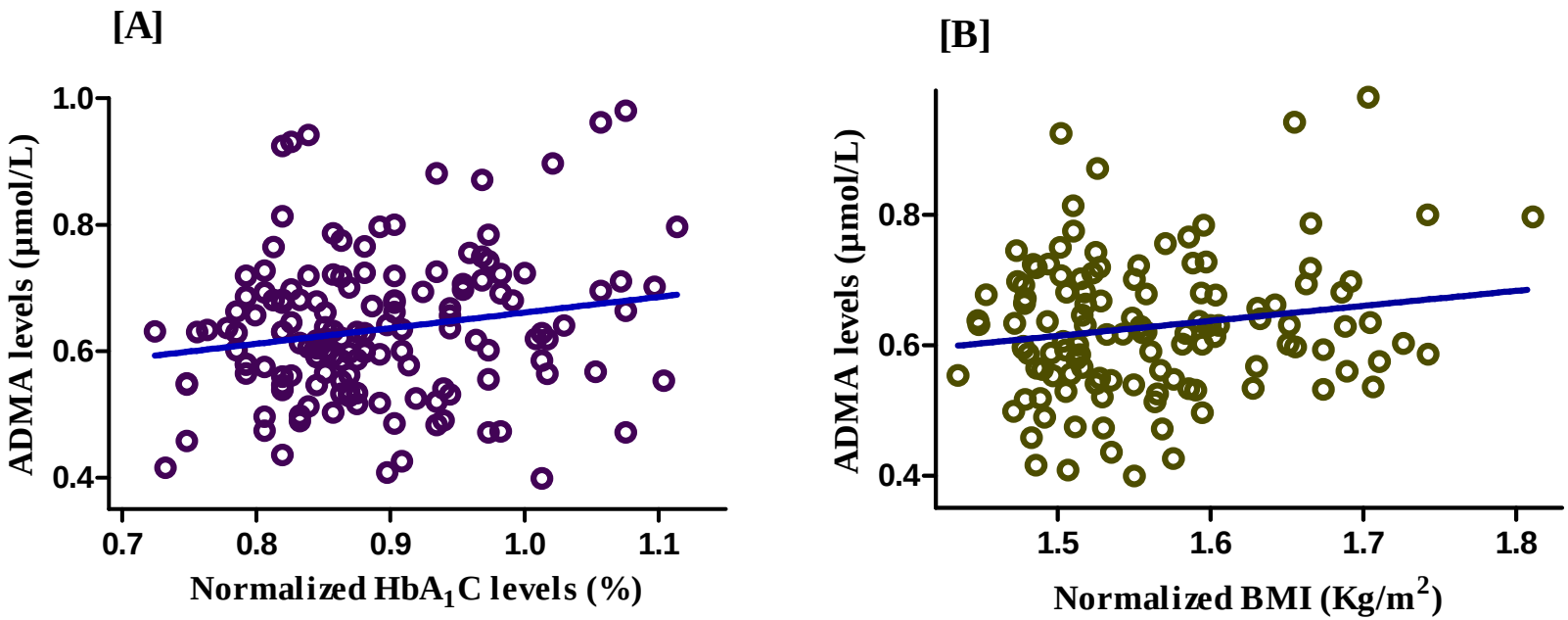

Figure 2.4 Correlation of ADMA concentrations with [A] HbA_1C (there was a positive correlation with ADMA levels: $\beta = 0.185$, $p = 0.025$); [B] BMI (there was a border-line positive correlation with ADMA levels: $\beta = 0.167$, $p = 0.06$) of the obese diabetics.

ADMA = asymmetric dimethylarginine; HbA_1C = glycated hemoglobin; BMI = body mass index.

2.5.3 Multivariate analyses

2.5.3.1 Determinants of 25(OH)D_3 levels

Multiple linear regression analysis was carried out to determine the independent correlates of 25(OH)D_3 level in these obese diabetic patients. Backward multiple linear regression analyses (Table 2.7), corrected for age, gender, BMI, corrected CrCL, hs-CRP, PTH, ADMA concentration, treatment with statins and ACEI/ARB or metformin and CV risk factors (hypertension and hypercholesterolemia)indicated the independent correlates of 25(OH)D_3 level were BMI ($\beta = -0.191$, $p = 0.039$) and ACEI/ARB treatment ($\beta = 0.192$, $p = 0.038$).

Table 2.7 Variables independently associated with 25(OH)D_3 levels (nmol/L) of obese-diabetic patients after backward multiple linear regression analysis

	β coefficient	**Significance (p value)**
BMI (Kg/m^2)	-0.191	0.039*
ACEI/ARB treatment	0.192	0.038*

BMI = body mass index, ACEI/ARB = angiotensin converting enzyme inhibitor or angiotensin receptor blocker.

* $p<0.05$.

2.5.3.2 Determinants of PTH levels

Multiple linear regression analysis was carried out to determine the independent correlates of PTH level in these obese diabetic patients. Backward multiple linear regression analyses (Table 2.8), corrected for age, gender, BMI, hs-CRP, treatment with statins and ACEI/ARB, season blood was collected and CV risk factors (hypertension and hypercholesterolemia) showed that the independent correlates of PTH level were: 25(OH)D_3 level ($\beta = -0.280$, $p = 0.001$), corrected creatinine clearance (CrCL) level ($\beta = -0.244$, $p = 0.006$) and ADMA concentration ($\beta = 0.196$, $p = 0.027$).

Table 2.8 Variable independently associated with elevated PTH levels (pmol/L) of obese-diabetic patients after backward multiple linear regression analysis

	β coefficient	**Significance (p value)**
ADMA (µmol/L)	0.196	0.027*
25(OH)D_3 (nmol/L)	-0.280	0.001*
Corrected CrCL (mL/min/1.73 m^2)	0.248	0.007*

ADMA=asymmetric dimethylarginine, CrCL= Creatinine clearance, 25(OH)D_3 = 25-hydroxyvitamin D_3. * $p<0.05$

2.5.3.3 Determinants of ADMA levels

Multiple linear regression analysis was carried out to determine the independent correlates of ADMA concentration in these obese diabetic patients. The backward multiple linear regression analyses (Table 2.9), corrected for age, gender, BMI, corrected CrCL, hs-CRP, 25-(OH)D_3 levels, treatments with statins and ACEI/ARB or metformin, season blood was collected and CV risk factors (hypertension and hypercholesterolemia) found that the independent correlates of ADMA concentration were PTH ($\beta = 0.278$, $p = 0.003$) and HbA_1C level ($\beta = 0.248$, $p = 0.007$).

Table 2.9 Variable independently associated with elevated ADMA levels (µmol/L) of obese-diabetic patients after backward multiple linear regression analysis

	β coefficient	**Significance (p value)**
BMI (Kg/m^2)	0.176	0.056
PTH (pmol/L)	0.278	0.003*
HbA_1C (%)	0.248	0.007*

BMI = body mass index, PTH = parathyroid hormone, HbA_1C = glycated hemoglobin.

* $p<0.05$

2.6 Discussion

The results of this study, which was conducted in non-institutionalized obese diabetic patient cohort, showed that there were no correlations on both univariate or multivariate analyses between plasma ADMA concentrations and 25(OH)D_3 levels. However, there was a robust relationship between ADMA concentrations and circulating PTH levels, independent of vitamin D status and concomitant cardiovascular risk factors.

Vitamin D deficiency and secondary hyperparathyroidism, either in combination or independently, have been associated with increased cardiovascular (CV) events. Replacement of vitamin D levels in vitamin D deficiency (Harris et al. 2011; Jablonski et al. 2011; Sugden et al. 2008; Tarcin et al. 2009) or correction of PTH levels in primary/secondary hyperparathyroidism (Kosch et al. 2000; Osto et al. 2012; Schillaci, Giuseppe et al. 2011; Vaziri et al. 1998) have been reported to improve the endothelial function and reduce CV events. ADMA (Section S1.3) is a competitive inhibitor of endothelial nitric oxide synthase (eNOS), and considered as a marker and mediator of endothelial dysfunction and biomarker of increased CV morbidity and mortality. Small increases in plasma ADMA levels can be responsible for several-fold higher coronary risk and cardiovascular mortality (Horowitz & Heresztyn 2007; Schnabel et al. 2005; Valkonen et al. 2001). Furthermore, ADMA has been considered as an independent predictor of coronary artery disease and cardiovascular death in diabetic patients as well as normal healthy individuals (Böger et al. 2009; Krzyzanowska et al. 2007; Meinitzer et al. 2007; Mittermayer et al. 2006; Schulze et al. 2006).

In previous studies, it has been shown that elevated PTH levels have also been linked to increased cardiovascular events. Several lines of evidence have suggested that disturbances in vitamin D and PTH metabolism have been associated with increased cardiovascular risk through diverse pathways (Block et al. 2004; Levin et al. 2006; Pirro, M et al. 2012; Sherman, Hollis & Tobin 1990). Elevated levels of PTH as a result of either primary hyperparathyroidism or secondary hyperparathyroidism have been found to be associated with increased cardiovascular mortality and morbidity (Block et al. 2004; Hagström et al. 2009; Palmer et al. 2011; Rostand & Drüeke 1999; Slinin, Foley & Collins 2005; Yu et al. 2010) independent of age, BMI, systolic blood pressure, diabetes, smoking, total cholesterol, HDL cholesterol, history of CVD and treatments with lipid-lowering and anti-hypertensive drugs. Plasma levels of PTH have also been reported to predict cardiovascular mortality even in the individuals with PTH levels within the normal reference levels (PTH < 6.8 pmol/L)

(Hagström et al. 2009). Elevated levels of PTH has been associated with an increased risk of hypertension (Rubin et al. 2005; Smith et al. 2000) and impaired glucose tolerance (Tassone et al. 2009) in the patients with primary hyperparathyroidism (PHPT). Furthermore, patients with PHPT have impaired insulin sensitivity (Chiu et al. 2000; Kumar et al. 1994), elevated LDL-cholesterol levels and decreased HDL-cholesterol levels (Hagström et al. 2002), and elevated circulating inflammatory markers such as hs-CRP and TNF-α levels independent of vitamin D status, and renal function (Øgard et al. 2005). In addition, PHPT patients have also been reported to have increased risk of congestive heart failure (Siu et al. 2007; Vestergaard et al. 2003). Importantly, removal of hyperparathyroid stimuli (by parathyroidectomy) has been reported to improve cardiovascular risk (Kosch et al. 2000; Osto et al. 2012; Schillaci, Giuseppe et al. 2011; Vaziri et al. 1998), indicating that normalizing PTH status is important for cardiovascular homeostasis.

Very few studies have reported the link between PTH and ADMA levels. In hemodialysis patients, plasma ADMA levels were significantly correlated with PTH levels, but this relationship was not corrected for vitamin D levels. Our group has previously published an inverse relationship between 25-hydroxyvitamin D3 (25-(OH)D3) concentrations and ADMA concentrations. The study was conducted in an ageing population and low 25-hydroxyvitamin D3 (25-(OH)D3) concentrations were associated with increased concentrations of ADMA (Ngo, D.T. et al. 2010). However, it was not determined whether this relationship was independent of PTH. In obese diabetic patients, with numerous concomitant cardiovascular diseases, we did not observe a relationship between ADMA and vitamin D. It is entirely possible that these obese diabetic patients already have elevated ADMA concentrations and a higher percentage of vitamin D deficient subjects. Therefore, this may have masked the inverse relationship between vitamin D and ADMA in these patients. On the other hand, the current study identified a positive relationship between ADMA levels with PTH concentrations, independent from vitamin D levels, renal function, cardiovascular risk factors and medicinal treatment with metformin, statins and angiotensin converting enzyme inhibitors/angiotensin receptor blockers (ACEI/ARB) in obese diabetics with an increased prevalence of vitamin D inadequacy (74%). A study conducted in patients with end-stage renal disease who were on hemodialysis, reported a similar significant positive correlation between PTH levels and ADMA concentrations, but this relationship was not corrected for vitamin D levels in these patients (Coen et al. 2009).

In this study, as predicted, the prevalence of vitamin D insufficiency (25(OH)D3 ≤ 75nmol/L) was high, approximately 75%, while majority of patients had PTH levels within normal range (60%). Therefore, the relationship between PTH and ADMA concentrations in this cohort exist even in obese/diabetic patients without severe renal impairment and with PTH levels mostly within the normal range. These results suggest that there is a close nexus between PTH and ADMA concentrations, even within the physiological PTH range.

Vitamin D levels are reported to vary by season in previous studies (Barnett, De Looper & Fraser 2008; Ngo, D.T. et al. 2010; van der Mei et al. 2007) and this pattern is reflected by the results of this study; 25(OH)D_3 levels of obese-diabetics in summer (mean±STD) (71.94±25.21 nmol/L) and autumn (68.84±20.33 nmol/L) are higher when compared to the levels in winter (57.50±22.40 nmol/L) and spring (51.21±23.53 nmol/L). The overall mean 25(OH)D_3 level for the participants is 60.6 ± 24.2 nmol/L, where the majority (67%) of the blood was taken during the spring and summer. This level was considered to be low since 25(OH)D_3 levels are reported to vary by season and 25(OH)D_3 levels are usually higher during the spring and summer with higher levels of solar UV radiation exposure in a population-based sample of the Australian population (van der Mei et al. 2007).

In this study, we also observed that a high plasma ADMA level is associated with high HbA_1C. Previous studies have demonstrated an association between glycaemic control and the prevalence of atherosclerosis and coronary artery disease in diabetic patients (Grundy et al. 1999; Laakso 1999; Selvin et al. 2004). The results of this study suggest that glycaemic control in obese diabetic patients may be a predictor of coronary vascular events. Poor glycaemic control is associated with increased oxidative risk and inflammation in obese diabetics (Esposito et al. 2002; Giugliano, Ceriello & Paolisso 1996). The obese diabetic patients of this study showed a significant positive association between glycaemic control (measured by HbA_1C and random glucose levels) and inflammation (measured by hs-CRP levels). High inflammatory activation was shown in the obese diabetic subjects with poor glycaemic control. The hyperglycaemia-induced oxidative stress has been shown to cause inactivation of ADMA clearance enzyme, dimethylarginine dimethylamino hydrolase (DDAH, Section 1.4) and could be a reason for the positive association between glycaemic control (HbA_1C) and higher ADMA concentrations.

The robust relationship we observed between PTH and ADMA concentrations should be observed with some caution because of the cross-sectional nature of the study design.

Furthermore, a study in healthy adults with varying age and health conditions should be conducted to determine whether the relationship between PTH and ADMA is only unique in obese diabetic patients. In addition, an intervention study to determine whether change in PTH levels also correlate with change in ADMA concentrations over time.

In conclusion, the prevalence of vitamin D deficiency and secondary hyperparathyroidism was high in free-living Australian obese-diabetic subjects. Increased PTH levels were associated positively with ADMA concentrations in obese diabetic subjects, independent of vitamin D status and may explain potential mechanisms for incremental coronary risk associated with raised PTH concentrations in epidemiological studies. In this population, glycaemic control measured by HbA_1C was an independent correlate of ADMA elevation. The relationship between poor diabetic control and ADMA elevation is consistent with previous reports (Abbasi et al. 2001) and might reflect redox stress-induced inhibition of the ADMA clearance enzyme, dimethylarginine dimethylamino hydrolase (DDAH) (Section S1.4). It is also possible that PTH concentrations may be useful as an additional biomarker of increased CV risk. The exact mechanism(s) underlying the PTH and ADMA association are uncertain and need to be evaluated directly both in the obese diabetic population and in healthy subjects.

Chapter 3 Effects of vitamin D_3 supplementation and change of PTH status on plasma concentrations of asymmetric dimethylarginine in healthy working adults

3.1 Abstract

Introduction: Vitamin D deficiency is increasingly prevalent in the general population and considered to be an important risk factor for the development of cardiovascular diseases including coronary artery disease/atherosclerosis. The effects of vitamin D deficiency and vitamin D supplementation on vascular endothelial function have also been demonstrated by various studies. The definite mechanisms which underline the associations are unclear. Our group has previously demonstrated an association between low 25(OH)D_3 levels with increased plasma levels of asymmetric dimethylarginin (ADMA), a competitive antagonist of endothelial nitiric oxide synthase (eNOS). This current study was designed to assess whether correction of vitamin D levels with vitamin D supplementation in vitamin D deficient but otherwise healthy subjects affects plasma ADMA concentrations. In our previous study (Chapter 2), we demonstrated that increased PTH level was a predictor of high ADMA concentration in obese-diabetic subjects, independent of vitamin D status. Thus, in this study, we also sought to examine whether the change in PTH levels with vitamin D supplementation correlate with the change in ADMA concentrations after vitamin D supplementation.

Methods and results: Healthy working adults (n=35) (age 45±10.8 years) (BMI 28.1±5.6 Kg/m^2) (mean±SD), without any pre-existing CV disease or diabetes, with vitamin D insufficiency (25-hydroxyvitamin D_3 levels (25(OH)D_3) < 75 nmol/L (30 ng/mL)) were recruited. Vitamin D administration (2000 IU/day for 12 weeks) significantly increased 25(OH)D_3 level from 48.8±16.5 nmol/L to 100.8±23.7 nmol/L, $p<0.001$ (mean±SD); significantly lowered serum PTH levels from 5.3±1.7 pmol/L to 4.7±1.9 pmol/L, $p<0.001$ and resulted in a borderline significant reduction in fasting glucose levels from 5.2±0.6 mmol/L to 5.1±0.6 mmol/L, $p = 0.053$. There were no changes in plasma ADMA concentrations ($p=0.59$) with the vitamin D supplementation. Furthermore, the vitamin D treatment significantly reduced both the mean systolic blood pressure levels (baseline 121±13 mmHg vs 118±14 mmHg, $p = 0.01$) and mean diastolic blood pressure levels (baseline 79±9 mmHg vs 77±8 mmHg, $p = 0.01$) of these subjects.

Plasma ADMA concentrations and hs-CRP levels did not correlate with $25(OH)D_3$ levels at baseline. Conversely, ADMA concentrations correlated with PTH levels at baseline and changes in ADMA concentrations were significantly correlated with changes in PTH levels ($\beta = 0.347$, $p = 0.041$) after vitamin D supplementation. Changes in ADMA concentrations after the vitamin D replacement was not correlated with changes in $25(OH)D_3$ levels ($\beta = 0.300$, $p = 0.08$).

Conclusion: In healthy young volunteers, vitamin D supplementation did not improve plasma ADMA or hs-CRP concentrations. However, changes in ADMA concentrations with vitamin D supplementation correlated significantly with the lowering of PTH levels. These results indicate that there may be a relationship between PTH and ADMA independent of vitamin D.

3.2 Introduction

Vitamin D deficiency is a rising pandemic worldwide with health consequences and is present in approximately 30% to 50% of the general population (Holick, M.F. 2007; Lee, J.H. et al. 2008). Recently, vitamin D deficiency has been found to be associated with increased cardiovascular mortality and morbidity (Lee, J.H. et al. 2008). However, the mechanism of this relationship is not known. One possible mechanism could be the effect of vitamin D on endothelial function (Ngo, D.T. et al. 2010; Sugden et al. 2008; Tarcin et al. 2009). Recently, a few studies have demonstrated the significant relationship between vitamin D and endothelial function. Importantly, these studies demonstrated the effects of vitamin D supplementation on the improved function of the endothelium (Sugden et al. 2008; Tarcin et al. 2009). However, the evidence available on the beneficial effects of vitamin D on endothelial function is inconsistent as some studies showed no effect (Witham et al. 2010)[,](Witham, M et al. 2012). Our group has demonstrated in an ageing population (n=254), low 25(OH)D_3 levels are associated with increased plasma concentrations of a methylated arginine residue, asymmetric dimethylarginine (ADMA) (Ngo, D.T. et al. 2010), a physiologically occurring competitive antagonist of eNOS, which is both a strong marker and also a mediator of many aspects of endothelial dysfunction (Boger 2003; Boger et al. 1997; Sydow & Munzel 2003) and increased hs-CRP, a marker of inflammation (Ngo, D.T. et al. 2010). This relationship persisted even after adjustments for season (Ngo, D. T. et al. 2010), age, body mass index (BMI) and other cardiovascular risk factors.

In the previous chapter, we showed that there was a robust significant relationship between elevated ADMA levels and PTH, independent of vitamin D. Indeed, elevated PTH has been shown to be associated with increased adverse cardiovascular events, via numerous possible mechanisms (as described in Chapter 1). Therefore, in this study, we sought to assess whether correction of vitamin D levels with vitamin D supplementation in vitamin D insufficient but otherwise healthy subjects affects plasma ADMA concentrations. Given our findings regarding the relationship between ADMA and PTH in the previous chapter, the natural progression to the previous study is to evaluate whether the change in PTH levels with vitamin D supplementation correlates with the change in ADMA concentrations upon vitamin D supplementation in healthy adults.

3.3 Aims and hypotheses of the study

In this clinical intervention study with vitamin D supplementation we investigated;

1. whether the correction of vitamin D levels with vitamin D supplementation in vitamin D insufficient but otherwise healthy subjects affects plasma ADMA concentrations.

2. whether the change in PTH levels with vitamin D supplementation correlates with the change in ADMA concentrations in this cohort of vitamin D insufficient ($25(OH)D_3$ < 75 nmol/L) but otherwise healthy adults.

Thus, we hypothesized that in vitamin D insufficient but otherwise healthy subjects:

1. vitamin D replacement reduces the plasma ADMA concentrations.
2. changes in PTH levels with vitamin D supplementation correlates positively with the change in ADMA concentrations.

3.4 Study protocol

All of the work in this study including interviewing the study participants and their recruitment, measurement of biological markers including ADMA, data collection, data entry and maintenance of database and statistical data analysis was performed by myself.

The threshold used for vitamin D insufficiency was 25-dihydroxyvitamin D_3 levels ($25(OH)D_3$) < 75 nmol/L. The subject cohort used in the study was healthy working adults from The Queen Elizabeth hospital (TQEH), South Australia. An invitation to participate was sent to all the staff attached to the hospital and according to the responses, sixty-five (n=65) healthy volunteers without any pre-existing heart disease, diabetes mellitus or liver / renal insufficiency were randomly screened for vitamin D insufficiency. The study was approved by the of Human Research Ethics Committee of TQEH and the University of South Australia. A written consent was obtained from each participant before inclusion in the study.

Thirty-five (n=35) subjects were identified with vitamin D insufficiency ($25(OH)D_3$ < 75 nmol/L). These subjects were randomized for the intervention study. The clinical history of each of these subjects was evaluated by a qualified medical practitioner before being

acceptedinto the study. None of the subjects were on vitamin D supplementation or diagnosed for any cardiovascular (CV) disease or diabetes at the time of recruitment.

The selected 35 subjects (30 females and 5 males) were given vitamin D supplementation (through the internal pharmacy of the TQEH) for 12 weeks and study parameters were measured at the baseline and 12 weeks after at a follow-up visit. The baseline data were collected during the spring (October - December) and the follow-up data were collected after 12 weeks during the Summer (January - March). Height, weight, and body mass index (BMI) were recorded for all the subjects at the study entry and follow up visits. Each subject was given a questionnaire to complete at each visit for evaluating their sun-exposure during the treatment period (Appendix E: Enrolment visit form and sun exposure questionnaire). At the study entry, physiological measures and fasting blood samples were collected for measuring biochemical parameters. Vitamin D_3 capsules were dispensed (in three containers x 60 capsules (total 180 capsules)) from the hospital pharmacy, 2000 IU dose of vitamin D_3 (two capsules of 1000 IU strength) daily prescribed for 12 weeks. At the end of the 12 weeks of the treatment period, the subjects were re-evaluated for the same parameters. The subjects were asked to return the empty containers and remaining capsules at the follow-up visit to evaluate compliance to the study treatment. The compliance to the treatment was calculated using remaining capsules count in the formula given below:

$$\textbf{Compliance \%} = \frac{(\text{Number of capsules dispensed} - \text{Quantity of capsules remaining}) \times 100}{(\text{Prescribed number of capsules per day}) \times (\text{Number of days between dispensing and re-visit dates})}$$

3.4.1 Biochemical and physiological measures tested at this stage

Fasting blood was taken via an antecubital vein for the determination of routine biochemical parameters. Biochemical and physiological parameters measured in the study can be summarized as follows:

3.4.1.1 Physiological measures

a. Resting blood pressure: systolic and diastolic blood pressure levels were obtained at the baseline and follow-up visit after 3 months. All blood pressure measurements were taken by a certified research nurse using standardized procedures and instruments, in the right arm, with a conventional mercury sphygmomanometer, after the participant was seated and rested. Three blood pressure measurements were taken 5 minutes apart and average of the three measurements were used for the analyses.

3.4.1.2 Biochemical measures

a. Plasma concentrations of ADMA, a marker and mediator of endothelial dysfunction (Böger, R.H. 2003), SDMA and arginine were determined by high-performance liquid chromatography with the derivatization reagent AccQ-Fluor (Waters, Milford, MA, USA) after solid phase extraction, as previously described (Heresztyn, Worthley & Horowitz 2004). The measurement of ADMA concentrations were carried-out by myself at the Cardiology Research Laboratory, Basil Hetzil Institute for Translational Health Research, Adelaide, South Australia. This method has been described in detail in section 2.4.2 (f).

b. Fasting glucose and fasting insulin levels were measured and homeostatic model assessment (HOMA) was calculated with regard to insulin resistance, as previously described (Katsuki et al. 2001).

c. 25(OH)D_3 levels were measured by liquid chromatography-mass spectroscopic (LC-MS/MS) method. This assay was validated and carried out by SA Pathology, Frome road, Adelaide, South Australia and the details of the method is attached (Appendix B). This method for assessment of vitamin D_3 (25(OH)D_3) levels was different to the method used in Chapter 2 for detecting the vitamin D_3 levels of obese-diabetic patient cohort. This was because the laboratory changed the method of vitamin D_3 analysis during the study. However, patient data comparison from the laboratory demonstrated a strong correlation between the chemiluminescence (IDS-isys 25-OHD) assay used in Chapter 2 and LC-MS/MS method used in this Chapter, with a Passing-Bablok fit of IDS-isys 25-OHD = 1.03, LC-MS/MS -6.53, $R^2 = 0.82$ (Cluse et al. 2012).

d. PTH (intact PTH) levels were measured by commercially available electrochemi luminescence immunoassay method (Roche Diagnostics, Indianapolis, IN). The assay was carried out by SA Pathology, Frome road, Adelaide, South Australia and the detailed method is attached (Appendix C).

e. High-sensitive C-reactive protein (hs-C-reactive protein) concentrations, as a marker of systemic inflammatory activation, lipid profile, creatinine, total serum calcium and ionised calcium were measured by a 1251 radio-immunoassay (Immunodiagnostic Systems Ltd, Bolden, UK). The assay was carried out by SA Pathology, Frome road, Adelaide, South Australia.

f. Renal function was assessed by creatinine clearance. The creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault equation (Cockcroft & Gault 1976) and indexed for body surface area with the Dubois and Dubois formula (DuBois & DuBois 1915).

g. Liver function tests were carried out to evaluate liver dysfunction (bilirubin, aminotransferases, and alkaline phosphatase levels were measured).

3.4.2 Statistical analyses

All data are expressed as mean ± standard deviation (mean±SD) unless otherwise stated. Normal distribution was tested for all continuous variables and skewed data were normalized by either log or square root transformations. Baseline and end-of-study parameters were compared by paired t-test (for normally distributed data) or Wilcoxon matched-pair signed-rank test (for non-parametric data). All t-tests were two-sided. Correlations between transformed, continuous, non-parametric data were made with linear regression. Changes of 25-hydroxyvitamin D_3, and changes of PTH levels during the treatment period were correlated to changes of ADMA, platelet nitric oxide responsiveness, hs-CRP, fasting glucose and insulin, HOMA, systolic and diastolic blood pressure levels and other study parameters by utilizing linear regression analysis. Determinants of the baseline ADMA, platelet nitric oxide responsiveness, hs-CRP, fasting glucose and insulin, HOMA, systolic and diastolic blood pressure levels were evaluated utilizing univariate analyses. All analyses were

performed with SPSS version 21 software (SPSS, Chicago, IL, USA), and a *P*-value of < 0.05 was considered to be statistically significant. All graphs/figures were generated with GraphPad Prism version 6 software (GraphPad Software, Inc., San Diego, California, USA).

Power calculation: Thirty-five (35) participants with 25-(OH)D_3 level < 75 nmol/L were enrolled into an open labelled vitamin D supplementation study. The sample size was determined according to the following calculation.

The mean plasma ADMA concentrations in a healthy cohort of women volunteers were 0.51±0.1 µmol/L. Thirty three (33) subjects are required to have 80% power to detect 0.5 SD difference induced by the treatment that is a change of 0.05 µmol/L. Considering the possible drop-out/non-compliance of approximately 2 participants, estimated to recruit 35 participants into this intervention study.

3.5 Results

3.5.1 Baseline subject characteristics

The study population (5 males and 30 females) consisted of 86% female subjects. The relative compliance to the study treatment remained high with mean±SD of 98±7 % (80-106%). The data collected for evaluating the sun-exposure during the treatment are given in Table 3.1. Walking and gardening were recorded as most common outdoor activities of the study subjects.

The study population was relatively young with 45±10.8 years (mean±SD) of age ranging from 22 years to 58 years. As previously described, all subjects had vitamin D insufficiency (25-(OH)D_3 < 75 nmol/L), but mean PTH levels were within previously described normal range (0.8-5.5 pmol/L). The renal and liver functions were well preserved. Calcium and phosphate levels were within the normal ranges. Mean plasma ADMA concentrations were within the previously reported normal range (mean value for the healthy population = 0.4 to 0.6 µmol/L)(Horowitz & Heresztyn 2007) for the methodology used in the laboratory.

Table 3.1 Comparison of the subject's sun-exposure

	Baseline (hours) Median (Min – Max) n=35	**Follow-up (hours) Median (Min – Max) n=35**	**Significance *P* value**
Total indoor work per week (hours)	37.5 (8 – 50)	37.5 (20 - 65)	0.06
Total outdoor hours per week (hours)	5.0 (0.0 – 20.0)	6.7 (0.0 – 23.0)	0.18

Max = Maximum; Min = Minimum; n=number of subjects.

The period of sun exposure and indoor working hours of these patients did not change significantly during the treatment period. The total outdoor activity time of some of the participating subjects remained at zero for the follow-up visit. A summary of the subject's baseline clinical characteristics are shown in Table 3.2.

Table 3.2 Baseline clinical characteristics of vitamin D insufficient healthy subjects (n=35)

Baseline Parameter	**mean±SD**	**Minimum - Maximum**
Age (Years)	45±10.8	22 - 58
BMI (Kg/m^2)	28.1±5.6	20 – 43
Vitamin D (25-$(OH)D_3$) level (nmol/L)	48.8±16.5	17 – 74
PTH level (pmol/L)	5.3±1.7	2.4 – 9.3
hs-CRP (mg/L)	3.7±5.5	0.3 – 27.0
Fasting Glucose (mmol/L)	5.2±0.6	4.3 – 7.2
Fasting insulin (mU/L)	12.9±7.2	3.4 – 33.0
HOMA-IR	3.0±1.7	0.7 – 7.1
Serum creatinine (µmol/L)	65.2±12.0	40.0 – 92.0

Baseline Parameter	mean±SD	Minimum - Maximum
CrCL (indexed for BSA) (mL/min/1.73 m^2)	96.4±16.1	71.0 – 127.5
Ionised calcium (mmol/L)	1.2±0.03	1.14 – 1.27
Total calcium (mmol/L)	2.4±0.08	2.19 – 2.50
Phosphate (mmol/L)	1.12±0.18	0.68 – 1.53
Calcium phosphate product $(mmol/L)^2$	2.65±0.50	1.49-3.69
Total cholesterol (mmol/L)	5.43±0.97	4.0 – 8.0
LDL (mmol/L)	3.2±0.8	2.0 – 5.3
HDL (mmol/L)	1.5±0.4	1.0 – 2.5
Total triglycerides (mmol/L)	1.6±0.96	0.4 – 5.0
Total cholesterol : HDL ratio	3.8±1.2	2.1 – 7.6
ADMA level (µmol/L)	0.56±0.09	0.42 – 0.82
SDMA level (µmol/L)	0.51±0.08	0.37 – 0.76
Arginine level (µmol/L)	94.76±18.13	57.33 – 142.17
Mean systolic blood pressure (mmHg)	121.4±13.3	102 - 160
Mean diastolic blood pressure (mmHg)	78.9±9.4	62 - 100

BMI = body mass index; hs-CRP = hs-C-reactive protein; HOMA-IR = homeostatic model assessment-insulin resistance; CrCL = creatinine clearance; LDL = low-density lipoproteins; HDL = high-density lipoproteins; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine.

3.5.1.1 Correlates of baseline vitamin D (25(OH)D_3) levels

Univariate analyses between 25(OH)D_3 levels versus continuous variables are documented in Table 3.3. In this cohort, ADMA (β = -0.020, p = 0.91), SDMA (β = 0.245, p = 0.16) or arginine (β = 0.112, p = 0.52) levels were not correlated with baseline 25(OH)D_3 levels.

Vitamin D_3 (25(OH)D_3) levels showed a significant positive correlation with age in these healthy volunteers (β = 0.363, p = 0.03). Fasting glucose levels showed a borderline positive correlation with 25(OH)D_3 levels (β = 0.339, p = 0.051) but fasting insulin (β = -0.133, p = 0.45) and HOMA-IR (β = -0.0.059, p = 0.74) levels were not. Serum phosphate levels showed a borderline positive correlation with plasma 25(OH)D_3 levels (β = 0.330, p = 0.053). Furthermore, plasma vitamin D levels showed a borderline positive correlation with calcium-phosphate product (β = 0.291, p = 0.09). The baseline vitamin D_3 levels showed a borderline inverse significance with baseline total triglycerides (β = -0.334, p = 0.050), but after the vitamin D replacement, the association was no longer significant (β = -0.174, p = 0.32). Other lipid components in this healthy cohort were not associated with baseline vitamin D levels.

Table 3.3 Univariate correlates between baseline 25(OH)D_3 levels and continuous variables of vitamin D insufficient healthy subjects

Baseline Parameter (n=35)	**Standardized coefficient (β)**	**Significance (p)**
Age (Years)	0.363	0.03*
BMI (Kg/m^2)	0.135	0.44
PTH level (pmol/L)	-0.237	0.17
hs-CRP (mg/L)	0.181	0.30
Fasting Glucose (mmol/L)	0.339	0.05 (0.051)
Fasting insulin (mU/L)	-0.133	0.45
HOMA-IR	-0.059	0.74
Serum creatinine (μmol/L)	0.113	0.52

Baseline Parameter (n=35)	Standardized coefficient (β)	Significance (p)
CrCL (indexed for BSA) (mL/min/1.73 m^2)	-0.379	0.02*
Ionised calcium (mmol/L)	0.228	0.19
Total calcium (mmol/L)	0.06	0.73
Phosphate (mmol/L)	0.330	0.05 (0.053)
Calcium phosphate product $(mmol/L)^2$	0.291	0.09
Total cholesterol (mmol/L)	-0.048	0.78
LDL (mmol/L)	0.045	0.80
HDL (mmol/L)	0.069	0.69
Total triglycerides (mmol/L)	-0.334	0.05 (0.050)
Total cholesterol : HDL ratio	-0.179	0.30
ADMA level (µmol/L)	-0.020	0.91
SDMA level (µmol/L)	0.245	0.16
Arginine level (µmol/L)	0.112	0.52
Mean systolic blood pressure (mmHg)	0.005	0.98
Mean diastolic blood pressure (mmHg)	-0.157	0.37

BMI = body mass index; hs-CRP = hs-C-reactive protein; HOMA-IR = homeostatic model assessment-insulin resistance; CrCL = creatinine clearance; LDL = low-density lipoproteins; HDL = high-density lipoproteins; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine.

* $p<0.05$

3.5.1.2 Correlates of baseline PTH levels

Univariate analyses between PTH levels versus continuous variables are documented in Table 3.4. Significant correlates with PTH levels are summarized in Figure 3.1. Of note, there was a very strong significant positive correlation with plasma ADMA levels ($\beta = 0.479$, $p = 0.004$), but not with the other methylated arginine residue, SDMA ($\beta = 0.298$, $p = 0.08$) or arginine ($\beta = 0.201$, $p = 0.25$). Other positive correlates of the baseline PTH level are age ($\beta = 0.335$, $p = 0.05$) and fasting glucose level ($\beta = 0.356$, $p = 0.04$). Phosphate level ($\beta = -0.477$, $p = 0.004$) and calcium-phosphate product level ($\beta = -0.455$, $p = 0.006$) were negatively correlated with PTH level in this cohort. However, in these healthy volunteers $25(OH)D_3$ ($\beta = -0.237$, $p = 0.17$), ionised calcium ($\beta = -0.241$, $p = 0.16$), total calcium ($\beta = -0.218$, $p = 0.21$) and hs-CRP ($\beta = -0.088$, $p = 0.62$) levels were not correlated with baseline PTH level. Baseline PTH level in this study cohort has shown a borderline positive correlation with baseline mean systolic blood pressure ($\beta = 0.296$, $p = 0.08$) and significant positive correlation with mean diastolic blood pressure ($\beta = 0.448$, $p = 0.007$).

Table 3.4 Univariate correlates between baseline PTH levels and continuous variables of vitamin D insufficient healthy subjects

Baseline Parameter (n=35)	**Standardized coefficient (β)**	**Significance (p)**
Age (Years)	0.335	0.05 (0.049)*
BMI (Kg/m^2)	0.117	0.50
$25(OH)D_3$ level (nmol/L)	-0.237	0.17
hs-CRP (mg/L)	-0.088	0.62
Fasting Glucose (mmol/L)	0.356	0.04*
Fasting insulin (mU/L)	0.141	0.42
HOMA-IR	0.203	0.24
Serum creatinine (μmol/L)	0.098	0.57

Baseline Parameter (n=35)	Standardized coefficient (β)	Significance (p)
CrCL (indexed for BSA) (mL/min/1.73 m^2)	-0.060	0.73
Ionised calcium (mmol/L)	-0.241	0.16
Total calcium (mmol/L)	-0.218	0.21
Phosphate (mmol/L)	-0.477	0.004*
Calcium phosphate product $(mmol/L)^2$	-0.455	0.006*
Total cholesterol (mmol/L)	0.070	0.688
LDL (mmol/L)	0.002	0.99
HDL (mmol/L)	-0.218	0.21
Total triglycerides (mmol/L)	0.311	0.07
Total cholesterol : HDL ratio	0.262	0.13
ADMA level (µmol/L)	0.479	0.004*
SDMA level (µmol/L)	0.298	0.08
Arginine level (µmol/L)	0.201	0.25
Mean systolic blood pressure (mmHg)	0.296	0.08
Mean diastolic blood pressure (mmHg)	0.448	0.007*

BMI = body mass index; hs-CRP = hs-C-reactive protein; HOMA-IR = homeostatic model assessment-insulin resistance; CrCL = creatinine clearance; LDL = low-density lipoproteins; HDL = high-density lipoproteins; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine.

* $p<0.05$

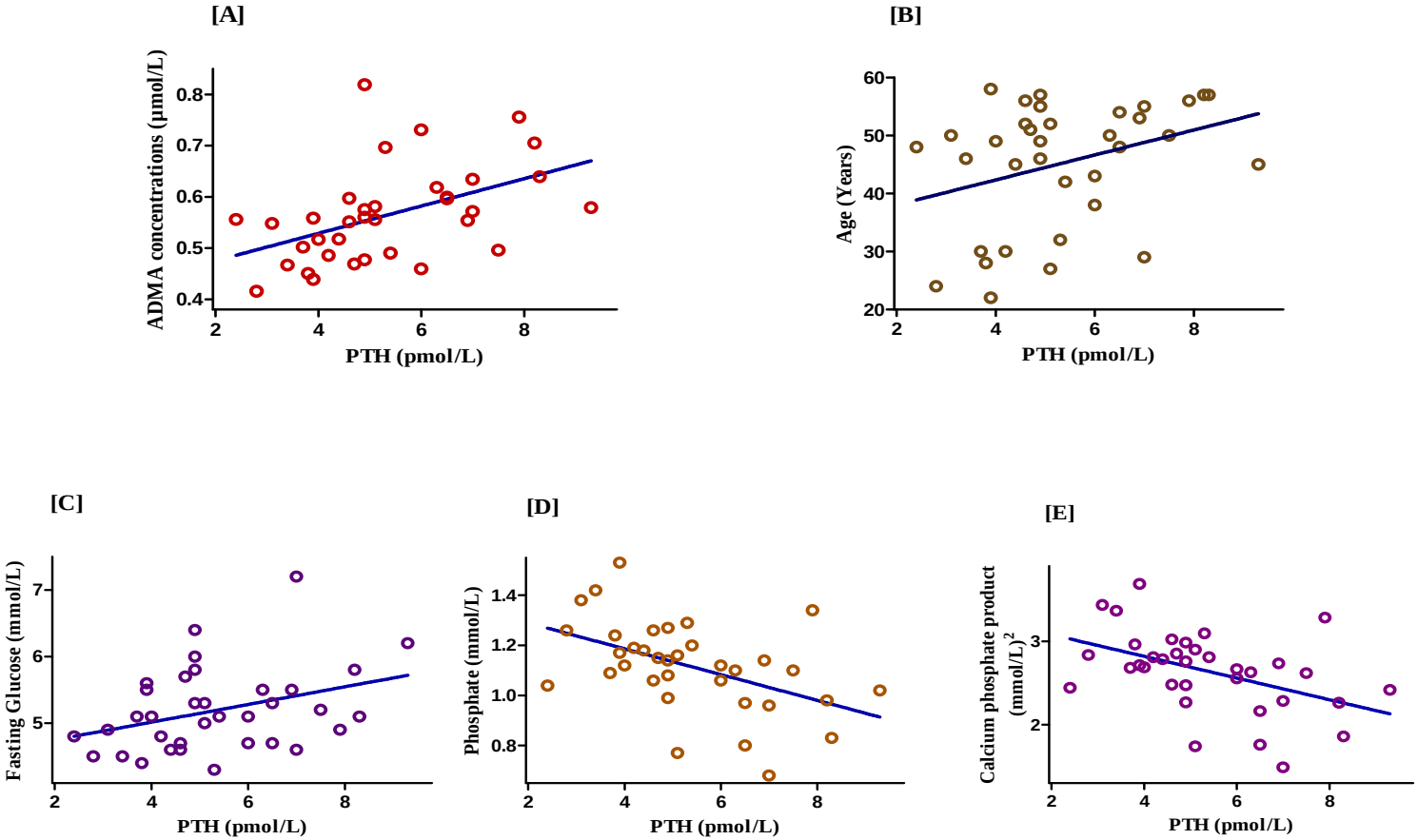

Figure 3.1 Correlation of PTH levels with [A] ADMA concentrations (there was an positive correlation with PTH levels: $\beta = 0.479$, $p = 0.004$); [B] Age (there was a positive correlation with PTH levels: $\beta = 0.335$, $p = 0.05$); [C] Fasting glucose levels (there was a positive correlation with PTH levels: $\beta = 0.356$, $p = 0.04$); [D] Phosphate levels (there was a negative correlation with PTH levels: $\beta = -0.477$, $p = 0.004$); [E] Calcium-phosphate product (there was a negative correlation with PTH levels: $\beta = -0.455$, $p = 0.006$) of healthy vitamin D insufficient subjects.

3.5.1.3 Correlates of baseline blood pressure levels

Baseline ADMA concentrations showed a significant positive association between both systolic blood pressure levels ($\beta = 0.328$, $p = 0.054$) and diastolic blood pressure levels ($\beta = 0.366$, $p = 0.030$). Furthermore, both systolic and diastolic blood pressure levels showed positive association with baseline PTH levels in these vitamin D insufficient healthy adults (Chapter 3, Section 3.5.1.2).

3.5.2 Change of parameters before and after vitamin D supplementation therapy

Changes in the study parameters at baseline and follow up visits of these randomly selected vitamin D insufficient healthy subjects are given in Table 3.5. Vitamin D administration for 12 weeks significantly increased 25-hydroxyvitamin D_3 levels compared with baseline values (48.8±16.5 nmol/L, baseline to 100.8±23.7 nmol/L, follow-up; $p < 0.001$) (Figure 3.2) and significantly reduced baseline PTH levels (5.3±1.7 pmol/L, baseline to 4.7±1.9 pmol/L, follow-up; $p<0.001$) to normal reference range (0.8-5.5 pmol/L). At the follow up visits 30 of 35 (86%) subjects increased their serum 25-hydroxyvitamin D_3 levels to the normal range (≥ 75 nmol/L). ADMA concentrations (0.56±0.09 µmol/L, baseline vs 0.57±0.08 µmol/L, follow-up, $p=0.59$) and hs-CRP levels (3.7±5.5 mg/L, baseline vs 3.6±4.7 mg/L, follow-up, $p=0.86$) did not change significantly with the vitamin D treatment for 3 months. Only borderline significant correlations were shown between the changes in vitamin D levels over the treatment period with changes in ADMA concentrations ($\beta = 0.300$, $p = 0.08$) and changes in corrected creatinine clearance levels ($\beta = 0.774$, $p = 0.07$). Furthermore, lipid profiles were not significantly changed after the vitamin D therapy. There were no significant differences in changes to the values of total calcium, ionised calcium, phosphate levels and calcium-phosphate product (Table 3.5). However, fasting glucose levels showed a borderline significant reduction after the vitamin D_3 treatment (5.2±0.6 mmol/L, baseline to 5.1±0.6 mmol/L, follow-up, $p=0.053$) (Figure 3.3).

However, changes in ADMA concentrations were significantly correlated with changes in PTH levels ($\beta = 0.347$, $p = 0.041$) after vitamin D supplementation for 12 weeks (Figure 3.4).

Vitamin D treatment significantly reduced both mean systolic blood pressure (121±13 mmHg, baseline to 118±14 mmHg, follow-up; $p = 0.01$) and mean diastolic blood pressure (79±9 mmHg, baseline to 77±8 mmHg, follow-up; $p = 0.01$) levels in this healthy subject

cohort (Figure 3.5). Elevation of vitamin D level was inversely correlated with differences in both systolic and diastolic blood pressure, $\beta = -0.445$, $p = 0.007$ and $\beta = -0.381$, $p = 0.024$ before and after the treatment, respectively.

In general, renal function and liver function were well preserved in this cohort and remained unchanged between the study visits. At the follow-up visit, no subject was found with either liver or renal dysfunction (corrected creatinine clearance < 30 mL/min/1.73 m^2).

Table 3.5 Changes of study parameters at baseline and follow-up visits of vitamin D insufficient healthy subjects

Baseline Parameter (n=35)	**Baseline (mean±SD)**	**Follow-up (mean±SD)**	***P* value**
Age (Years)	45 ± 10.8	45 ± 10.7	
BMI (Kg/m^2)	28.1 ± 5.6	27.6 ± 7.5	0.50
Vitamin D_3 (25-(OH)D_3) level (nmol/L)	48.8 ± 16.5	100.8 ± 23.7	<0.001**
Mean systolic blood pressure (mmHg)	121 ± 13	118 ± 14	0.01*
Mean diastolic blood pressure (mmHg)	79 ± 9	77 ± 8	0.01*
PTH level (pmol/L)	5.3 ± 1.7	4.7 ± 1.9	<0.001**
hs-CRP (mg/L)	3.7 ± 5.5	3.6 ± 4.7	0.86
Fasting Glucose (mmol/L)	5.2 ± 0.6	5.1 ± 0.6	0.05 (0.053)
Fasting insulin (mU/L)	12.9 ± 7.2	12.0 ± 6.9	0.18
HOMA-IR	3.0 ± 1.7	3.5 ± 4.9	0.27
Serum creatinine (μmol/L)	65.2 ± 12.0	64.7 ± 10.4	0.59
CrCL (indexed for BSA) (mL/min/1.73 m^2)	96.4 ± 16.1	96.5 ± 13.0	0.61
Ionised calcium (mmol/L)	1.2 ± 0.03	1.2 ± 0.03	0.39
Total calcium (mmol/L)	2.4 ± 0.08	2.3 ± 0.08	0.63

Baseline Parameter (n=35)	**Baseline (mean±SD)**	**Follow-up (mean±SD)**	***P* value**
Phosphate (mmol/L)	1.12 ± 0.18	1.09 ± 0.14	0.15
Calcium phosphate product $(mmol/L)^2$	2.65 ± 0.50	2.56 ± 0.36	0.22
Total cholesterol (mmol/L)	5.43 ± 0.97	5.30 ± 0.93	0.34
LDL (mmol/L)	3.2 ± 0.8	3.2 ± 0.96	0.67
HDL (mmol/L)	1.5 ± 0.4	1.47 ± 0.37	0.08
Total triglycerides (mmol/L)	1.6 ± 0.96	1.7 ± 1.14	0.12
Total cholesterol : HDL ratio	3.8 ± 1.2	3.8±1.2	0.62
ADMA level (µmol/L)	0.56 ± 0.09	0.57 ± 0.08	0.59
SDMA level (µmol/L)	0.51 ± 0.08	0.51 ± 0.08	0.65
Arginine level (µmol/L)	94.76 ± 18.13	97.10 ± 18.5	0.41

BMI = body mass index; CrCl = creatinine clearance; HOMA-IR = homeostatic model assessment-insulin resistance; LDL = low-density lipoproteins; HDL = high-density lipoproteins; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine;

* $p<0.05$, ** $p<0.001$

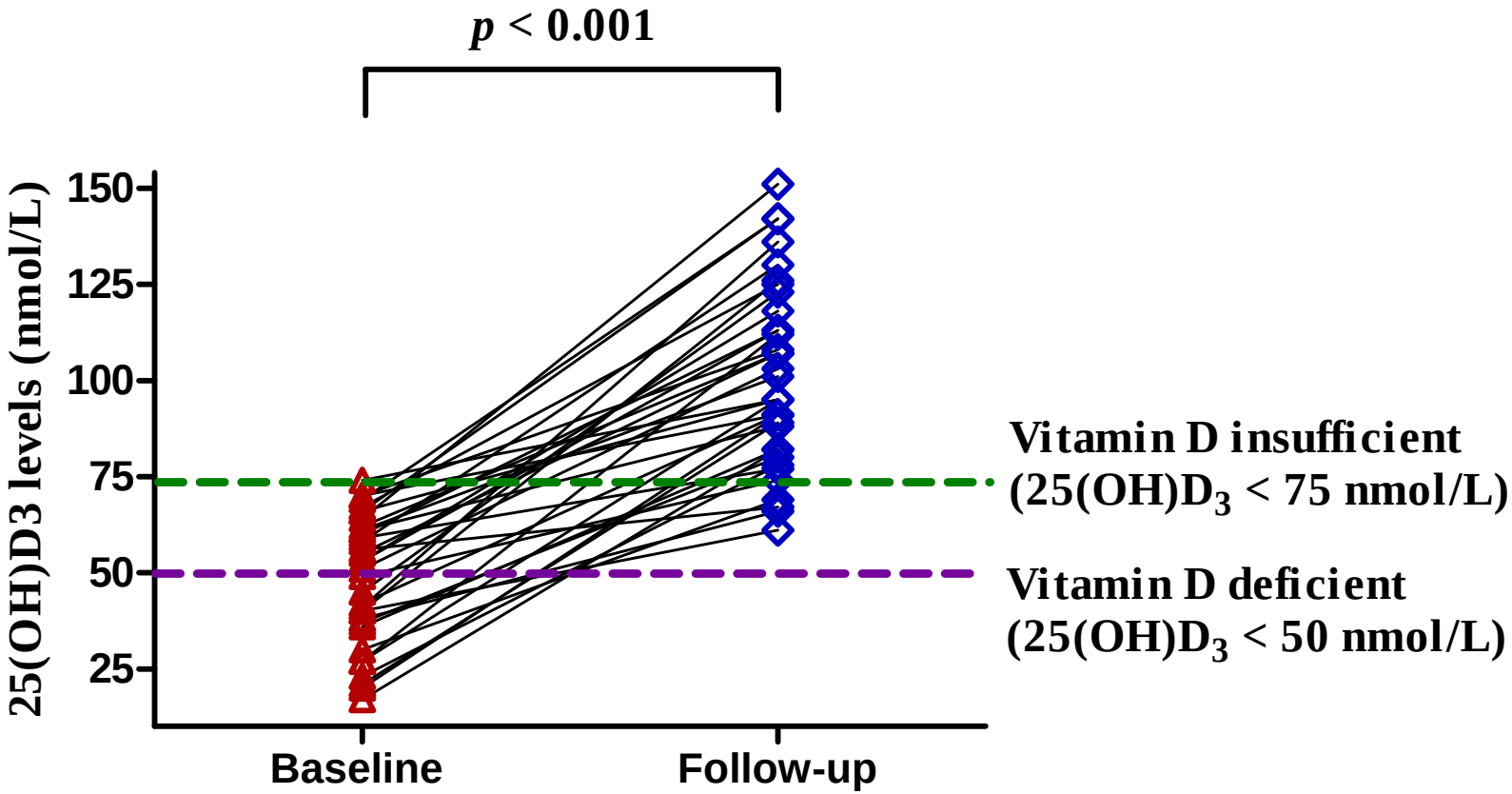

Figure 3.2 Individual changes in 25(OH)D_3 levels after the vitamin D_3 treatment ($p < 0.001$)

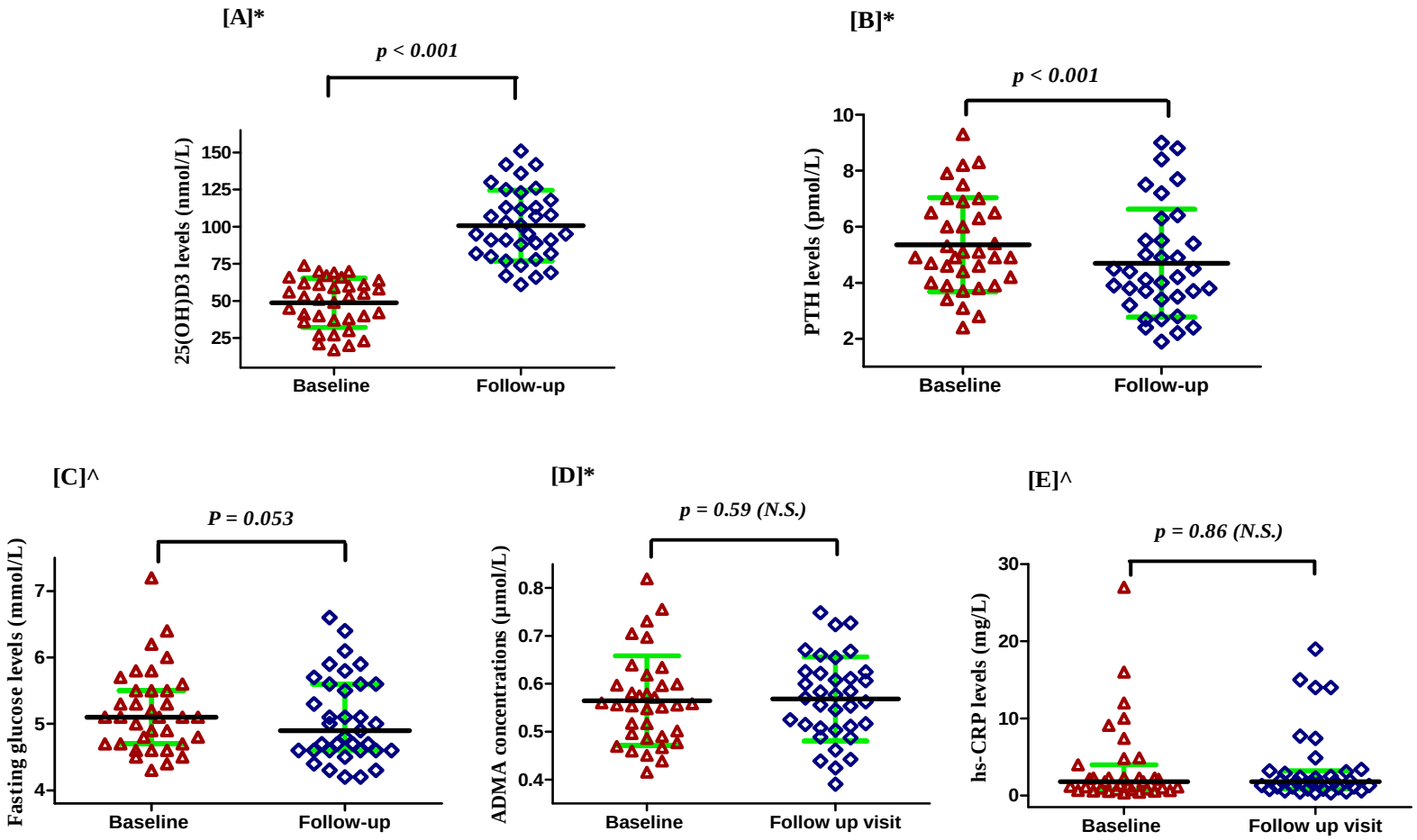

Figure 3.3 Changes of parameters with vitamin D_3 replacement therapy in healthy subjects

* indicates the mean±SD ^ indicates the median (interquartile range)

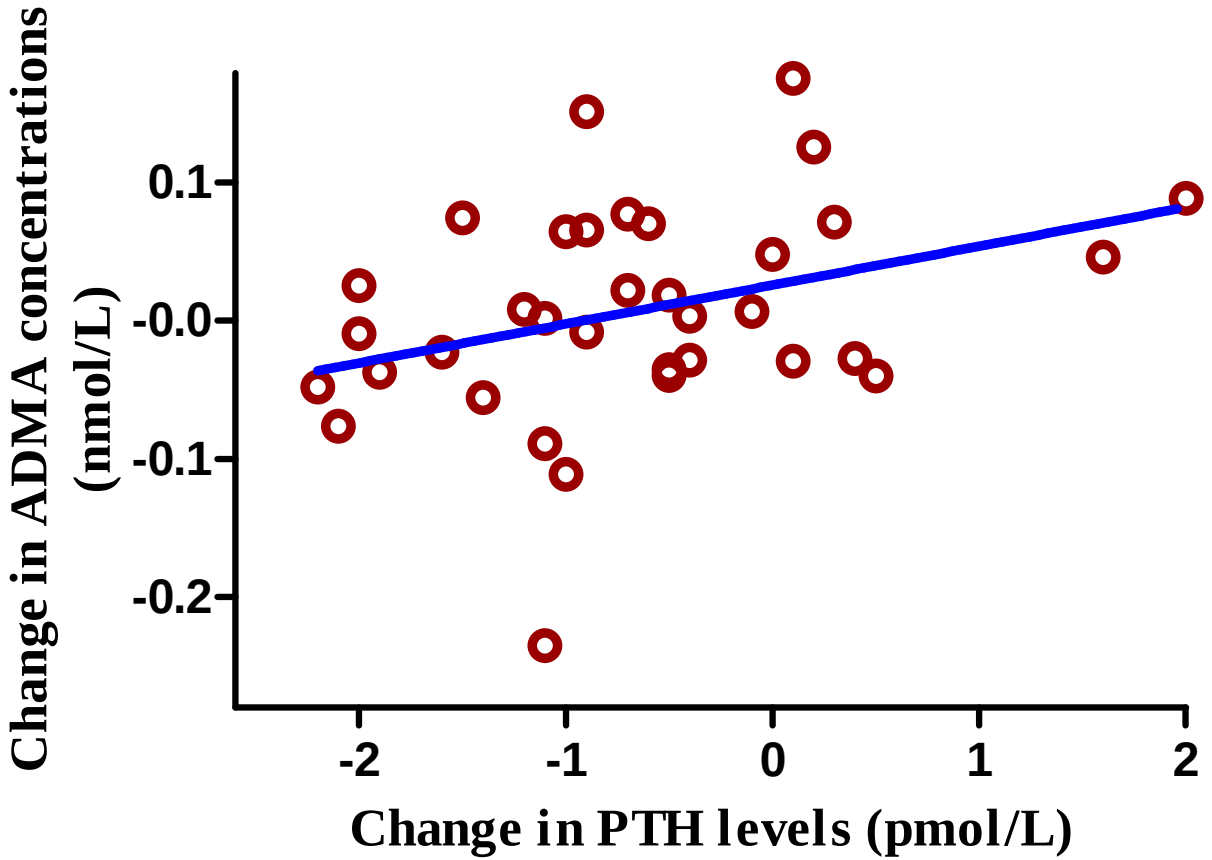

Figure 3.4 Correlation of changes in PTH levels with changes in ADMA concentrations (there was a positive correlation with PTH levels: $\beta = 0.347$, $p = 0.041$)

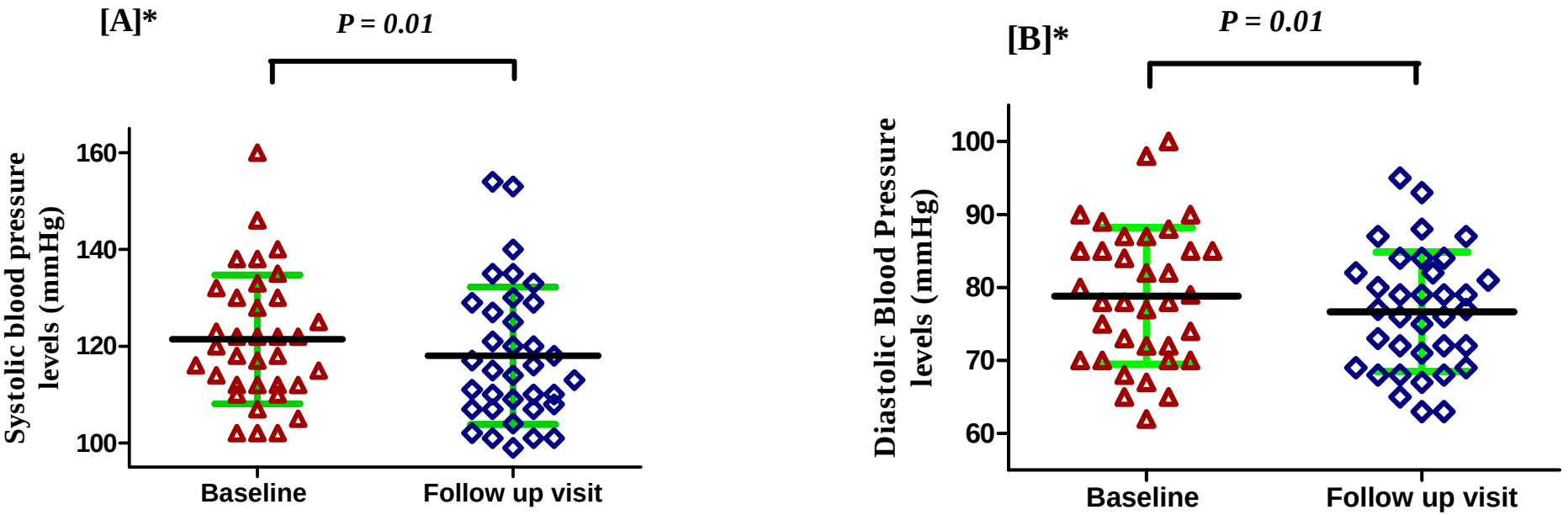

Figure 3.5 Significance mean changes of [A] Systolic blood pressure (121 ± 13 Vs 118 ± 14 mmHg, p = 0.01); [B] Diastolic blood pressure (79 ± 9 Vs 77 ± 8 mmHg, p = 0.01) with vitamin D_3 replacement therapy.

* indicates the mean±SD

3.6 Discussion

In this study, in healthy vitamin D insufficient volunteers, we found that vitamin D supplementation did not reduce plasma ADMA or hs-CRP concentrations. We used a vitamin D daily dose of 2000 IU for 12 weeks to correct the vitamin D levels of these healthy vitamin D insufficient subjects. The 2000 IU daily dose was selected as this is a dose recommended for achieving the most beneficial health effects of vitamin D and recognized as a safe by the U.S. Food and Drug Administration's nutritional guidelines (Lee, John H. et al. 2008).

The vitamin D replacement therapy of 2000 IU per day for 12 weeks significantly increased plasma 25(OH)D_3 levels in all subjects who had low baseline vitamin D levels. At the end of the study, 31 of 35 subjects were vitamin D sufficient (25(OH)$D_3 \geq 75$ nmol/L). The increase in 25(OH)D_3 levels after 12 weeks on 2000 IU per day was from 48.8 ± 16.5 nmol/L to 100.8 ± 23.7 nmol/L (Table 3.5). The increase in 25(OH)D3 levels of approximately 2-fold over 12 weeks is high compared to previous studies (Sugden et al. 2008; Tarcin et al. 2009). We recruited patients for baseline in autumn-spring seasons, and the follow-up period finished in spring-summer seasons. According to our previous findings (Ngo, D.T. et al. 2010), there is a significant seasonal fluctuations, and during spring-summer, there is significantly higher 25(OH)D3 levels versus autumn-winter seasons. Therefore, it is entirely possible that the rapid increase of 25(OH)D3 levels in this cohort is partially due to the change in season over the follow-up period. This coincides with a slight, albeit not significant, increase in the hours spent outdoors by the participants during the follow-up period (Table 3.1).

Vitamin D has been reported to exert protective effects on endothelial cell activity and inflammation/oxidative stress through several mechanisms (Kassi et al. 2013). Furthermore, it was demonstrated that vitamin D can stimulate NO production in VDR mediated, dose-dependent manner in human umbilical vein endothelial cell cultures through eNOS activation (Molinari et al. 2011; Queen et al. 2006). In vivo and in vitro experiments have demonstrated that a vitamin D analog (22-oxacalcitriol) significantly suppressed expression of NADPH oxidase, which is the major enzyme system responsible for generation of superoxide radicals in vasculature, thus reducing oxidative stress in endothelium (Hirata et al. 2013). Furthermore, a study demonstrated that vitamin D reduce oxidative stress by preventing the generation of superoxide anion generation (Polidoro et al. 2013). Vitamin D has been reported to modulate the vascular tone by regulating the release of vasoconstrictor

metabolites of arachidonic acid (Vanhoutte, Feletou & Taddei 2005). Calcitriol inhibits the expression of inflammatory cytokines (IL-6, IL-8) through inhibition of nuclear factor-κB to reduce inflammatory activation (Equils et al. 2006; Talmor et al. 2008a). Moreover, calcitriol has been reported to exert vascular protection by counteracting the harmful effects of advanced glycation end products. Increased levels of advanced glycation end products and glucose can induce the expression of pro-inflammatory molecules such as IL-6, IL-8 through nuclear factor-κB-mediated mechanisms (Zitman-Gal et al. 2012).

Clinically, as discussed in Chapter 1 (Section 1.2.5.2.4), vitamin D supplementation has been reported to improve vascular endothelial function. We did not observe a change in ADMA concentrations in our vitamin D insufficient, but otherwise healthy volunteers, despite significant improvement in vitamin D concentrations after supplementation. It is possible that subjects with poor vitamin D status at entry ($25(OH)D_3$ levels $\leq$ 30 nmol/L) could benefit more from vitamin D supplementation. Even though our subjects did not have any concomitant cardiovascular diseases at entry, we did observe that their plasma ADMA concentrations are higher than previously published for the same age group (Horowitz & Heresztyn 2007).

Effects of vitamin D on glucose and insulin profiles

Previous experimental studies have shown that vitamin D may have direct effects on pancreatic β cells (Kadowaki & Norman 1985; Lee et al. 1994). However, clinical studies have shown inconsistent results regarding the benefits of vitamin D supplementation on improving glucose and insulin profiles (Mathieu, Chantal et al. 2001; Pittas et al. 2006) (Section 1.2.5.2.1). Although our study was not powered to specifically examine these effects of vitamin D supplementation in this cohort, we observed some reduction in fasting glucose and a trend in lowering fasting insulin levels. Of note, our subjects were not diagnosed with diabetes upon entry of the study, though, there were 7 of 35 subjects with elevated fasting glucose and insulin levels (5.6-7.2 mmol/L). Although fasting glucose and insulin levels are clinically used as markers of insulin sensitivity, other measures such as insulin-clamp should be used to more accurately measure the effects of vitamin D on glucose and insulin profiles. It is also possible that vitamin D supplementation may have beneficial effects on glucose/insulin profiles in patients without multiple concomitant co-morbidities, and that vitamin D therapy should begin at a much earlier age to maintain adequate vitamin D levels

to potentially sustain normal glucose/insulin sensitivity over time. To date, there has been no adequately powered studies with appropriate vitamin D dosage designed to specifically look at the effects of vitamin D supplementation on glucose/insulin sensitivity over a long period of follow-up.

Effects of vitamin D on lipid profiles

Historically, observational studies have shown an inverse relationship between low $25(OH)D_3$ levels and elevated serum lipid profiles, including triglycerides (Lind et al. 1995; Martins et al. 2007a; Zittermann, A et al. 2009), and therefore suggest that the beneficial effects of vitamin D on the cardiovascular system is partly due to the effects of vitamin D on favorable lipid profiles. A recent review (Challoumas 2014) showed that in related randomized controlled trials (RCTs) identified in the literature from 1987 to present, out of 19 relevant RCTs identified, only one reported benefits of vitamin D supplementation on lipid profile parameters, while the rest showed no effects or even adverse outcomes, which are highlighted by the only meta-analysis in the field. Similarly, in this study, we observed a significant inverse relationship between baseline $25(OH)D_3$ and plasma triglyceride levels, vitamin D supplementation showed no effects on lipid/triglyceride profiles in our subjects. It has been suggested (Challoumas 2014) that the discordant results between observational and interventional studies regarding effects on lipids are a result of confounding lifestyle factors: those with high vitamin D levels are more likely to lead healthier outdoor lifestyles with better diets versus those with lower vitamin D levels.

Effects of vitamin D on blood pressure

In normotensive, healthy subjects, we observed a significant reduction in both systolic and diastolic pressure, with 3 months of vitamin D replacement therapy. As previously explained in Chapter 1 (Section 1.2.5.2.3), vitamin D has been shown to inhibit renin synthesis, and thus reduces angiotensin II levels. Furthermore, we also observed that low vitamin D levels is associated with the lack of use of ACEi/ARB in obese diabetics, independent of history of hypertension (Chapter 2, Section 2.5.2.1). It is entirely possible, that the effects on blood pressure seen with vitamin D supplementation are due to its effects on the renin-angiotensin system. Although, inconsistent, other studies have also shown beneficial effects of vitamin D

supplementation on blood pressure in human studies (Sugden et al. 2008; Tarcin et al. 2009). The inconsistent nature of the existing literature on blood pressure could be related to the different dosage regimes and patient cohorts with varying risk factors. Of note, our study was not originally powered to detect a difference in blood pressure in these subjects. Furthermore, 24-hour blood pressure monitoring may be a more accurate assessment of blood pressure status.

We also observed a positive correlation between PTH levels and blood pressure. The higher PTH status is associated with an elevated blood pressure. PTH has also been shown to activate the renin-angiotensin system, along with effects on aldosterone levels (Fischer et al. 2013). It was recently shown that upon adjustment for, age, sex, estimated glomerular filtration rate, serum 25-hydroxy vitamin D concentration, waist circumference, body mass index, hypertension, diabetes mellitus, liver disease, and intake of drugs that affect the RAAS or bone metabolism, serum PTH levels were positively correlated with circulating aldosterone-renin ratio in over 3000 subjects in the general population (Fischer et al. 2013). Therefore, it is entirely possible that the improvement in blood pressure seen in this study is partly due to a reduction in concomitant PTH-associated with the normalization of vitamin D status.

Relationship between change in ADMA and PTH levels with vitamin D supplementation

To date, there is no study which has documented a relationship between ADMA and PTH in subjects with normal renal functions. Previously, we observed a robust relationship between plasma ADMA and PTH levels in obese diabetic patients, independent of vitamin D status (Chapter 2, Section 2.5.3.2). In healthy adults with vitamin D insufficiency we also observed: 1) a significant relationship between baseline ADMA and PTH levels, and 2) the change in PTH levels correlated with the change in ADMA concentrations after 3 months of vitamin D supplementation. It is noteworthy, that PTH levels in both our obese-diabetics and healthy adults are mostly within normal range. Therefore, these data, to the best of our knowledge, showed for the first time that there is a strong nexus between PTH and ADMA.

Hyperparathyroidism has been associated with increased adverse cardiovascular events (Block et al. 2004; Levin et al. 2006; Pirro, M et al. 2012; Sherman, Hollis & Tobin 1990). Vascular endothelial dysfunction (Osto et al. 2012), elevated oxidative stress (Tanaka et al.

2011), and inflammation (Christensen et al. 2011) have been postulated to be the mechanisms for these observations (as reviewed in Chapter 1, Section 1.3). As mentioned previously, elevated ADMA is associated with endothelial dysfunction, and a biomarker of adverse cardiovascular events (Boger 2003; Boger et al. 1997; Sydow & Munzel 2003). The strong nexus between small elevations of PTH and increased ADMA concentrations observed in this study suggest that small increases of PTH could also be associated with increases in adverse cardiovascular outcomes by increasing ADMA concentrations.

However, this was not a randomized, placebo-controlled study, and the relationship between PTH and ADMA in healthy adults remains inconclusive. Furthermore, to account for temporal and seasonal effects of vitamin D status, future cross-over studies should be performed to better assess the effects of vitamin D supplementation and PTH on ADMA concentrations.

In conclusion, increased PTH level is a predictor of high ADMA concentration independent of vitamin D status and may explain potential mechanisms for incremental coronary risk associated with raised PTH concentrations in epidemiological studies. It is possible that PTH concentrations may be useful as an additional biomarker of increased CV risk or as a window on endothelial function.

Chapter 4 Vitamin D supplementation in healthy adults lowers plasma thrombospondin-1 levels: A novel vitamin D target?

4.1 Abstract

Introduction: Vitamin D deficiency is increasingly prevalent in the general population and is associated with cardio-metabolic dysfunction. Thrombospondin-1 (TSP-1), a stress-released matricellular protein is a potent inhibitor of the nitric oxide-cyclic GMP (NO-cGMP) signaling pathway in vascular cells and contributes to vascular endothelial dysfunction. In this study, we examined the relationship between vitamin D and plasma TSP-1 concentrations in healthy volunteers and the effects of vitamin D supplementation on plasma TSP-1 levels and other markers of NO signaling in vitamin D deficient subjects.

Methods and results: Among healthy volunteers (n=58) aged 23 to 71 years there was a significant inverse relationship between plasma vitamin D and TSP-1 concentrations. Multivariate analyses indicated that TSP-1 levels remained a significant predictor of low vitamin D levels (β= -0.3, p=0.035) independent of age and BMI. There was no relationship between vitamin D levels and other parameters for the integrity of the NO system or inflammation. In a separate cohort of otherwise healthy volunteers (n=35) with vitamin D insufficiency, a 12 week treatment period with 2000IU/daily of vitamin D3 resulted in a marked and significant reduction in plasma TSP-1 levels ($p<0.001$), systolic (p=0.007) and diastolic (p=0.008) blood pressure and almost complete normalization of plasma vitamin D levels (in 86% of subjects).

Conclusions: 1) Plasma TSP-1 levels vary inversely with plasma vitamin D levels in healthy adults; 2) Vitamin D supplementation in vitamin D-insufficient, but otherwise healthy, individuals leads to a significant reduction in plasma TSP-1 levels, which may have significant beneficial cardiovascular effects. Further evaluation of vitamin D supplementation in larger clinical trials is warranted to assess whether its effect in reducing TSP-1 levels translates into changes in cardiovascular outcomes.

4.2 Introduction

Vitamin D deficiency is common among healthy subjects (affects approximately 30% to 50% of the general population) (Holick, M.F. 2007; Looker et al. 2002) and contributes to the pathogenesis of numerous diseases.

In adults, Holick et al (Holick, M.F. 2007) reported that the prevalence of vitamin D deficiency is as high as 50%, while the Third National Health and Nutrition Examination Survey (NHANES) data reported its prevalence in the United States to be 25% to 57% of adults (Looker et al. 2002).

In the last 5 years, it has been increasingly suggested that vitamin D is important for cardiovascular homeostasis (Lee, J.H. et al. 2008; Temmerman 2011; Wang et al. 2012; Wang, T.J. et al. 2008), and conversely, that vitamin D deficiency may represent a risk factor for the development of coronary disease (Szeto et al. 2012; Zittermann, A. & Koerfer 2008). Vitamin D levels have been correlated both with various cardiovascular risk factors and events. To date, the mechanism(s) underlying the putative detrimental effects of vitamin D deficiency on cardiovascular function remain unclear. There is emerging evidence of a relationship between low vitamin D levels and impaired vascular nitric oxide (NO) homeostasis. Low vitamin D levels have been shown to be associated with increased plasma concentrations of asymmetric dimethyl arginine (ADMA) in aging population (Ngo, D. T. et al. 2010). ADMA is an endogenous competitive antagonist of eNOS, and biomarker of endothelial dysfunction.

Furthermore, the expression of thrombospondin-1 (TSP-1), an inhibitor of NO signaling, is significantly down regulated by vitamin D analogs in human aortic smooth muscle cells (Wu-Wong, J. R., Nakane, M. & Ma, J. 2006; Wu-Wong, Nakane & Ma 2007; Wu-Wong et al. 2006). To date, understanding of effects of vitamin D supplementation on surrogate biomarkers of vascular function and thrombogenicity is limited. The primary objectives of this study were to: 1) establish the relationship between vitamin D and plasma TSP- 1 levels in healthy volunteers, and 2) determine whether vitamin D supplementation will favorably affect biomarkers of nitric oxide signaling, in particular, TSP-1 and ADMA concentrations, and platelet responsiveness to the NO donor, sodium nitroprusside (SNP).

4.3 Part 1 of the study

4.3.1 Aims and hypotheses of the study

To date, no study has been performed to assess the possible relationship between vitamin D and TSP-1 despite some (limited) evidence in tissue culture models (Wu-Wong, J R, Nakane, M & Ma, J 2006). Therefore, the first aim of this study is to establish whether there is a relationship between circulating $25(OH)D_3$ levels and TSP-1 concentrations in a clinical setting. We hypothesized in the first part of the study that in healthy subjects without concomitant CV diseases, low $25(OH)D_3$ is associated with elevated concentrations of TSP-1.

4.3.2. Study protocol

The work in this study including measurement of biological markers including ADMA and TSP-1, data collection, data entry and maintenance of database and statistical data analysis was performed by myself.

4.3.2.1 Study Subjects

Healthy volunteers were consecutively recruited from advertisements placed in the North Western Adelaide area. A clinical history of each subject was evaluated by a qualified medical practitioner before enrolment to the study. None of the subjects were receiving vitamin D supplementation at the time of recruitment. The study was approved by the of Human Research Ethics Committees of The Queen Elizabeth Hospital and the University of South Australia. Written consent was obtained from each participant before their inclusion into the study.

4.3.2.1.1 Study cohort 1: Evaluation of the relationship between vitamin D and TSP-1 concentrations in healthy volunteers

Studies were conducted in healthy adult volunteers (81% female), (n=58), aged 23 to 71 years. All participants reported that they: a) were in good health, b) were not currently taking any medications for cardiovascular diseases, or c) did not have any known significant medical history. No subject was receiving vitamin D supplements. Subject characteristics are

described in Table 4.1. Relationships between plasma 25-hydroxyvitamin D_3 ($25(OH)D_3$) and TSP-1 concentrations were analyzed by univariate and multivariate analyses adjusted for age, body mass index (BMI), and gender.

4.3.2.2 Analyses common for the entire study

Fasting blood was taken via an antecubital vein for the determination of routine biochemical parameters. Biochemical and physiological parameters measured in the study can be summarized as follows:

4.3.2.2.1 Biochemical measures

a Plasma concentrations of TSP-1 were determined by ELISA (enzyme-linked immunosorbent assay) designed to measure the naturally occurring human thrombospondin-1 (Quantikine ELISA human thrombospondin-1 immunoassay kit, R & D systems, Minneapolis, MN, USA). All measurements of TSP-1 were carried-out by myself at the cardiology research laboratory, Basil Hetzil Institute for Translational Health Research, Adelaide, South Australia.

4.3.2.2.1.1 Thrombospondin-1 assay

In all patients, blood was collected into heparinised blood collection tubes and centrifuged at 4ºC and 1000 x g for 15 minutes within 30 minutes of collection. The resulting supernatant plasma was separated to a new Ependroff tube and an additional centrifugation step was carried out at 10,000 x g for 10 min at 4ºC for complete removal of the platelets. The supernatant plasma was stored at -80 ºC until assayed.

The "Quantikine ® ELISA Human TSP-1 assay kit" (R & D systems, Minneapolis, MN, USA) included the following materials:

- Thrombospondin-1 microplate (Part 893298) – 96 well polystyrene microplate (12 stripes of 8 wells) coated with a mouse monoclonal antibody against Thrombospondin-1.
- Thrombospondin-1 conjugate (Part 893299) – 21 mL of polyclonal antibody against Thrombospondin-1 conjugated to horseradish peroxidase with preservatives.

- Thrombospondin-1 standard (Part 893300) – 1000 ng of recombinant Thrombospondin-1 in a buffered protein solution with preservatives; lyophilized.
- Assay Diluent RD1-56 (Part 895102) – 17 mL of a buffered protein solution with preservatives.
- Calibrator Diluent RD5-33 Concentrate (Part 895813) – 21 mL vial of buffered protein base with preservatives. Dilute 20 mL of the solution into 20 mL of distilled water to prepare 40 mL of calibrator diluent RD5-33 (1x)
- Wash buffer concentrate (Part 895003) – 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives. Dilute 20 mL of the wash buffer concentrate into distilled water to prepare 500 mL of wash buffer.
- Colour reagent A (Part 895000) – 12.5 mL of stabilized hydrogen peroxide.
- Colour reagent B (Part 895001) – 12.5 mL of stabilized chromogen (tetramethylbenzidine).
- Stop solution (Part 895032) – 6 mL of 2 N sulphuric acid
- Instruction booklet

Sample preparation

Samples were removed from the freezer and thawed at room temperature. The thawed plasma samples were then diluted (5-fold dilution) by combining 100 µL of sample with 400 µL of calibrator diluent (RD5-33(X1), R & D Systems).

Standards preparation

The given TSP-1 standard (Part 893300) was reconstituted with 1.0 mL of distilled water and the resultant standard solution of 1000 ng/mL was used as a stock solution for preparing standard series. Eppendorf tubes (polypropylene) were used to make the standard series with the calibrator diluent by 1 in 2 dilution at each step. Standard concentration series of 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.3 ng/mL, 15.6 ng/mL and 7.81 ng/mL were prepared. The calibrator diluent serves as the zero standard (0 ng/mL).

Quality control of the assay

Analyte recovery for accuracy and precision was determined by spiking plasma from healthy volunteers with known quantities of TSP-1 (thrombospondin-human platelet, Athens research and technology, Inc. Athens, Georgia, USA). The standard stock was prepared by reconstituting 25 µg of the standard powder with 250 µL of distilled water to make a 100 µg/mL stock solution. Medium and high quality controls (QCs) were prepared by spiking the pooled healthy plasma as follows:

Low QC - Pooled plasma of 5 healthy volunteers served as low quality control.

Medium QC – 1995 µL of the pooled plasma was spiked with 5 µL of the standard stock.

High QC - 1990 µL of the pooled plasma was spiked with 10 µL of the standard stock.

The prepared quality control stocks were aliquoted to Eppendorf tubes and stored at -20 ºC until used. The quality controls were thawed prior to use and the low QC solution was diluted (1:5), medium QC (1:10) and high QC (1:10) with calibrator diluent (RD5-33(X1), R & D Systems).

Microplate assay of thrombospondin 1

TSP-1 microplates with 96 wells (Part 893298, R & D Systems) were used and assay diluent (RD1-56, R & D Systems) was added to each well before dispensing 50 µL of each sample, standard and QC sample. Triplicate measures were performed for each sample, standard and QC solution. The plates were incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm. After 2 hours the plate was aspirated with the wash buffer (x1) provided (Part 895003, R & D Systems). Each well was washed with the wash buffer (400 µL) four times and 200 µL of thrombospondin 1 conjugate (Part 893299, R & D Systems) added. The plate was then incubated for another 2 hours on the shaker as before and the aspiration step repeated before adding 200 µL of substrate solution (equal volumes of Part 895000 and 895001, R & D Systems) to each well. The samples were incubated again for 30 minutes at room temperature while protected from light. After 30 min 50 µL of the stop solution (Part 895032, R & D Systems) was used to end the reaction and the optical density of each well was measured within 30 minutes using a microplate reader (R & D systems, Minneapolis, MN, USA) set to a 450 nm reference wave length. A wave length correction

was also carried out by subtracting the optical density measured at the correction wave length of 540 nm.

Calculation of results

All triplicate readings were averaged and the values for each standard, sample and QC solution was subtracted from the value of the zero standard. The standard curve was created by using a four parameter logistic curve-fit (GraphPad Prism 5.03, GraphPad Software, Inc., La Jolla, CA 92037 USA) for each assay for the quantification of both the samples and quality controls.

b. 25(OH)D_3 levels were measured by a liquid chromatography-mass spectroscopic (LC-MS/MS) method. This assay was validated and carried out by SA Pathology, Frome road, Adelaide, South Australia and the details of the method are attached inAppendix B.

c. PTH (intact PTH) levels were measured by commercially available electrochemi luminescence immunoassay method (Roche Diagnostics, Indianapolis, IN). The assay was carried out by SA Pathology, Frome road, Adelaide, South Australia and the detailed method is attached in Appendix C.

d. Plasma concentrations of ADMA, a marker and mediator of endothelial dysfunction (Böger, R.H. 2003), SDMA and arginine were determined by high-performance liquid chromatography with the derivatization reagent AccQ-Fluor (Waters, Milford, MA, USA) after solid phase extraction, as previously described bu Heresztyn et al. (Heresztyn, Worthley & Horowitz 2004). All the measurements of plasma ADMA concentrations were carried-out by myself at the Cardiology Research Laboratory, Basil Hetzil Institute for Translational Health Research, Adelaide, South Australia. This method has been described previously in Chapter 2, Section 2.4.2 (f).

e. Fasting glucose and fasting insulin levels were measured and homeostatic model assessment (HOMA) was calculated with regard to insulin resistance, as previously described by Katsuki et al. (Katsuki et al. 2001).

f. High-sensitive C-reactive protein (hs-C-reactive protein) concentrations, as a marker of systemic inflammatory activation, lipid profile, creatinine, total serum calcium and ionised calcium were measured by a 1251 radio-immunoassay (Immunodiagnostic Systems Ltd, Bolden, UK).

g. Renal function was assessed by creatinine clearance. The creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault equation (Cockcroft & Gault 1976) and indexed for body surface area with the Dubois and Dubois formula (DuBois & DuBois 1915).

h. Liver function tests were carried out to evaluate liver dysfunction (bilirubin, aminotransferases, and alkaline phosphatase levels were measured).

4.3.2.2.2 Physiological measures

a Resting blood pressure (systolic and diastolic blood pressure) levels were obtained at the baseline and follow-up visit after 3 months. All blood pressure measurements were taken by certified research nurse using standardized procedures and instruments, in the right arm, with a conventional mercury sphygmomanometer, after the participant was seated and rested. Three blood pressure measurements were taken 5 minutes apart and average of the three measurements were used for the analyses.

b Platelet aggregation and platelet responsiveness to the NO donor SNP was assessed using whole-blood aggregometry with a dual channel impedence aggregometer (model 560, Chrono-Log, Havertown, Pennsylvania, USA) (Chirkov, Yuliy Y et al. 2002). All the assessments of platelet NO responsiveness were carried-out by myself at the Cardiology Research Laboratory, Basil Hetzil Institute for Translational Health Research, Adelaide, South Australia. The detailed method is described below.

4.3.2.2.2.1 Assessment of platelet nitric oxide responsiveness

Platelet aggregation and platelet responsiveness to the NO donor sodium nitroprusside (SNP) was assessed using whole-blood aggregometry with a dual channel impedence aggregometer (model 560, Chrono-Log, Havertown, Pennsylvania, USA) (Chirkov, Yuliy Y et al. 2002).

Sample preparation

Blood samples were collected using "Winged infusion sets" or "Butterfly infusion sets" to draw blood slowly without activating the platelets. The blood samples were kept on the bench-top at room temperature for 20 min before their dilution. Whole blood samples were diluted two-fold with normal saline (450 µL of whole blood in 500 µL of normal saline) and the resulting solution was pre-warmed at 37 ºC for 5 minutes before the test.

Platelet aggregation study

The method used in this laboratory has been described previously (Chirkov, Y.Y. et al. 1999; Chirkov, Yuliy Y et al. 2002) and was performed at 37 ºC with a stirring speed of 900 rpm.
In the ADP-induced aggregation study, aggregation was induced with adenosine 5'-diphosphate (ADP) (final concentration of 1 µmol/L). The platelet aggregation was monitored continually for 7 min and electrical impedence responses were recorded (RO-3 Rikadenki chart recorder) in ohms (Ω). The test was duplicated for each sample.
To analyse platelet responsiveness to NO, an exogenous NO donor, SNP was added to the samples at a concentration of 10 µmol/L one minute before adding the ADP. The platelet aggregation was monitored as described above and the test was duplicated for each sample.

Evaluation of the results

The ADP-induced aggregation was reported as an average of the duplicated readings.

Platelet responsiveness to exogenous NO (inhibition of platelet aggregation) was reported as a percentage of platelet responsiveness to SNP when compared to the platelet responsiveness

to ADP in the absence of SNP. An average of the duplicate readings was used in the calculations.

4.3.2.3 Statistical analyses

All data are expressed as mean ± SD unless otherwise stated. Normal distribution was tested for all continuous variables, and skewed data were normalized by either log or square root transformation. Baseline subject group characteristics between vitamin D insufficient and sufficient groups were compared utilizing non-paired t-test (for normally distributed data) or Mann-Whitney test (for non-parametric data). Baseline and end-of-study parameters were compared by paired t-test (for normally distributed data) or Wilcoxon matched-pair signed-rank test (for non-parametric data). All t-tests were two-sided. Correlations between transformed, continuous, non-parametric data were made with linear regression. Multivariate analysis (backward multiple linear regression analysis) was utilized to identify independent determinants of TSP-1 levels adjusted for BMI and age in subjects of study cohort 1. Variables included to predict elevated TSP-1 levels were age, BMI and vitamin D (25-(OH)D_3) levels. All analyses were performed with the SPSS version 21 software (SPSS, Chicago, IL, USA) and a *P*-value of < 0.05 was considered to be statistically significant. All graphs/figures were generated with GraphPad Prism version 6 software (GraphPad Software, Inc., San Diego, California, USA).

4.3.2.4 Results of the part 1 of the study (study cohort 1)

4.3.2.4.1 Evaluation of the relationship between vitamin D and TSP-1 concentrations in healthy volunteers

The baseline subject characteristics of study cohort 1 are displayed in Table 4.1.

Table 4.1: Clinical characteristics of healthy volunteers in cohort 1

Baseline Parameter (n=58)	mean±SD	Minimum - Maximum
Age (Years)	49±10	23-71
BMI (Kg/m^2)	27.7 ± 5.1	19.8 – 44.1
Vitamin D (25-(OH)D_3) level (nmol/L)	73.5 ± 25.5	28 - 137
TSP-1 (ng/mL)	153 ± 119	26 - 675
hs-CRP (mg/L)	2.8 ± 3.2	0.22 – 11.00
Fasting Glucose (mmol/L)	5.0 ± 1.8	3.5 – 17.9
Fasting insulin (mU/L)	8.2 ± 4.8	2.4 – 24
HOMA-IR	1.7 ± 1.1	0.48 – 5.8
Total cholesterol (mmol/L)	5.4 ± 0.9	4.0 – 9.4
LDL (mmol/L)	3.2 ± 0.9	1.0 – 7.0
HDL (mmol/L)	1.7 ± 0.4	0.9 – 2.4
Total triglycerides (mmol/L)	1.5 ± 1.2	1 - 8
ADMA level (µmol/L)	0.57 ± 0.08	0.49 - 0.76
SDMA level (µmol/L)	0.58 ± 0.13	0.43 – 0.91
Arginine level (µmol/L)	97.63 ± 26.55	46.40 – 156.35
ADP-induced platelet aggregation (Ω)	8.83 ± 2.95	2 – 14
% Inhibition of platelet aggregation	32.93 ± 28.16	0.0 – 100.0

BMI = body mass index; hs-CRP = hs-C-reactive protein; TSP-1 = thrombospondin-1; HOMA-IR = homeostatic model assessment-insulin resistance; CrCL = creatinine clearance; LDL = low-density lipoproteins; HDL = high-density lipoproteins; TSP-1 = thrombospondin-1; ADP = adenosine di-phosphate; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine.

4.3.2.4.2 Correlates of baseline vitamin D_3 ($25(OH)D_3$) levels in healthy volunteers

Univariate analyses between $25(OH)D_3$ levels versus continuous variables are documented in Table 4.2. There was a significant inverse relationship between $25(OH)D_3$ and TSP-1 concentrations (β=-0.282; P=0.047) (Figure 4.1), but not with plasma ADMA concentrations, platelet responsiveness to SNP, or hs-CRP (Table 4.2). Furthermore, there was a borderline significant inverse correlation between vitamin D levels and ADP-induced platelet aggregation (β=-0.309; P=0.055). On multivariate analysis, high TSP-1 levels remained a significant correlate of low $25(OH)D_3$ (β= -0.3, p=0.035), independent of BMI and age.

Table 4.2 Univariate correlates between baseline $25(OH)D_3$ levels and continuous variables of healthy volunteers in cohort 1

Baseline Parameter (n=58)	**Standardized coefficient (β)**	**Significance (p)**
Age (Years)	0.013	0.92
BMI (Kg/m^2)	-0.15	0.91
TSP-1 (ng/mL)	-0.282	0.047*
hs-CRP (mg/L)	-0.170	0.21
Fasting Glucose (mmol/L)	0.010	0.94
Fasting insulin (mU/L)	-0.277	0.08
HOMA-IR	-0.239	0.13
Total cholesterol (mmol/L)	-0.183	0.17
LDL (mmol/L)	-0.128	0.38
HDL (mmol/L)	0.223	0.13
Total triglycerides (mmol/L)	-0.204	0.16
ADMA level (µmol/L)	0.208	0.42
SDMA level (µmol/L)	0.132	0.61
Arginine level (µmol/L)	0.018	0.94

Baseline Parameter (n=58)	Standardized coefficient (β)	Significance (p)
ADP-induced platelet aggregation (Ω)	-0.309	0.05 (0.055)
% Inhibition of platelet aggregation	0.178	0.24

BMI = body mass index; hs-CRP = hs-C-reactive protein; TSP-1 = thrombospondin-1; HOMA-IR = homeostatic model assessment-insulin resistance; CrCL = creatinine clearance; LDL = low-density lipoproteins; HDL = high-density lipoproteins; TSP-1 = thrombospondin-1; ADP = adenosine di-phosphate; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine.

* $p<0.05$

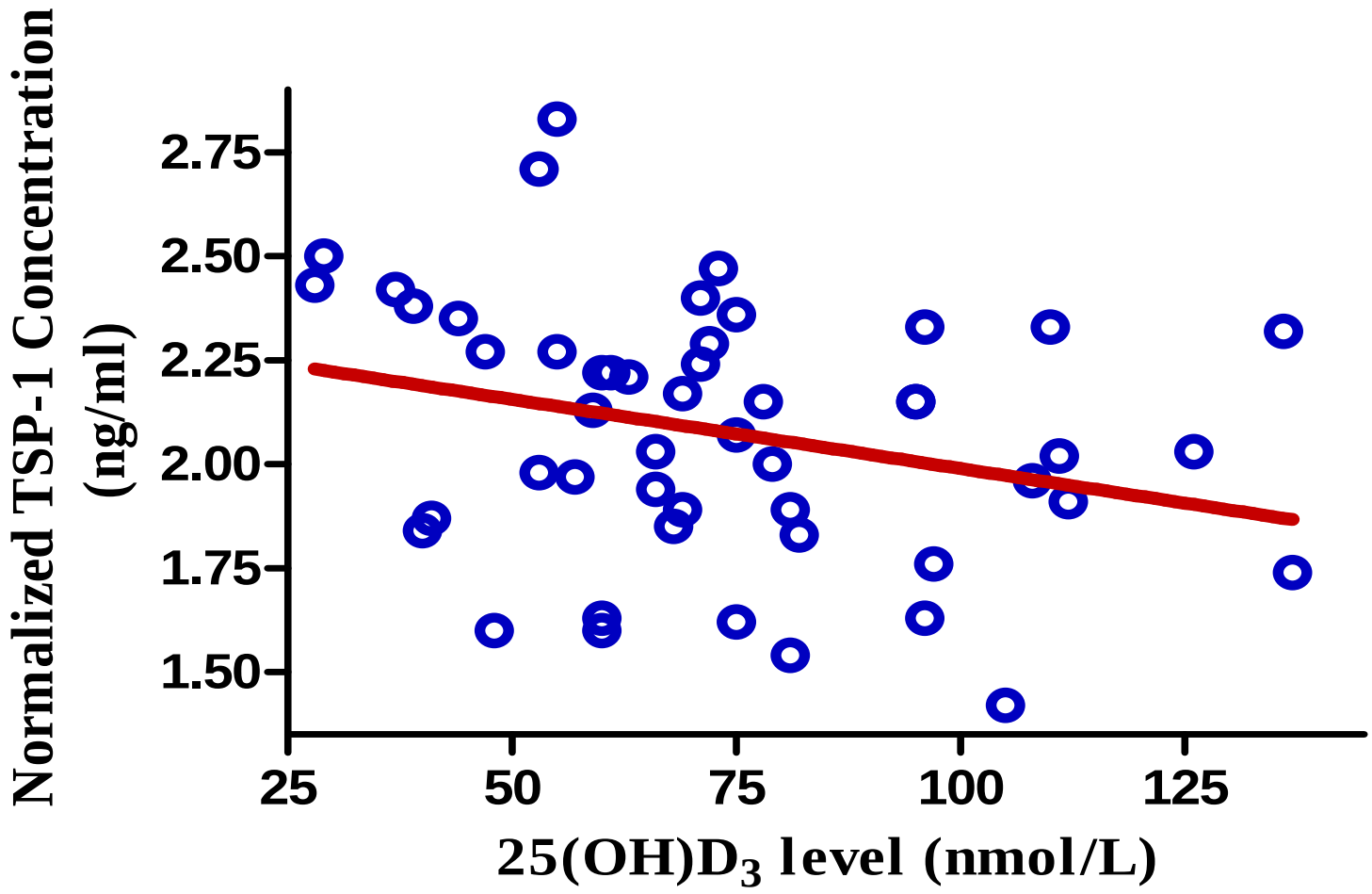

Figure 4.1 Correlation of TSP-1 levels with 25(OH)D_3 levels (there was a significant negative correlation with TSP-1 levels $\beta = -0.282$, $p = 0.047$) in healthy subjects (study cohort 1).

4.3.2.4.3 Multivariate analyses - Determinants of TSP-1 levels

Multiple linear regression analysis was carried out to determine the independent correlates of the TSP-1 level in these healthy subjects (study cohort 1). Backward multiple linear regression analyses (Table 4.3), corrected for age and BMI showed that $25(OH)D_3$ level remained as an independent correlate of TSP-1 concentration ($\beta = -0.285$, $p = 0.045$).

Table 4.3 Variables independently associated with TSP-1 levels (ng/mL) of healthy volunteers (study cohort 1) after backward multiple linear regression analysis

	β coefficient	**Significance (p value)**
$25(OH)D_3$ level (nmol/L)	-0.285	0.045*
BMI (Kg/m^2)	-0.236	0.096

BMI = body mass index, $25(OH)D_3$ = 25-hydroxyvitamin D_3

* $p<0.05$.

4.4 Part 2 of the study

4.4.1 Study cohort 2: Evaluation of the effects of vitamin D supplementation in healthy volunteers (This study was conducted as a sub-study of the interventional study described in chapter 3).

Given our findings above, that 25(OH)D_3 levels were inversely correlated with plasma TSP-1 concentrations in healthy adults, in part 2 of the study, we sought to determine whether supplementation of vitamin D in vitamin D deficient, but otherwise healthy adults would lower TSP-1 concentrations.

Thirty-five healthy working volunteers without any pre-existing heart disease, diabetes mellitus or renal insufficiency were screened for vitamin D insufficiency, defined as plasma 25-hydroxyvitamin D_3 levels < 75 nmol/L (Holick, M. F. 2007).

Height, weight, and body mass index (BMI) were recorded for all the subjects at the study entry and at the follow up visit. Fasting blood samples were collected for the biochemical parameters described below. Vitamin D_3 capsules (Ostelin® vitamin D capsules (Australia)) were dispensed from the hospital pharmacy at a dose of 2000 IU of vitamin D_3 (two capsules of 1000 IU) daily for 12 weeks. At the end of the 12 weeks of treatment, the subjects were re-evaluated for the same parameters. The baseline data were collected during the spring (October - December) and the follow-up data were collected after 12 weeks during the Summer (January - March). The consort flow diagram of the study is illustrated in Figure 4.2.

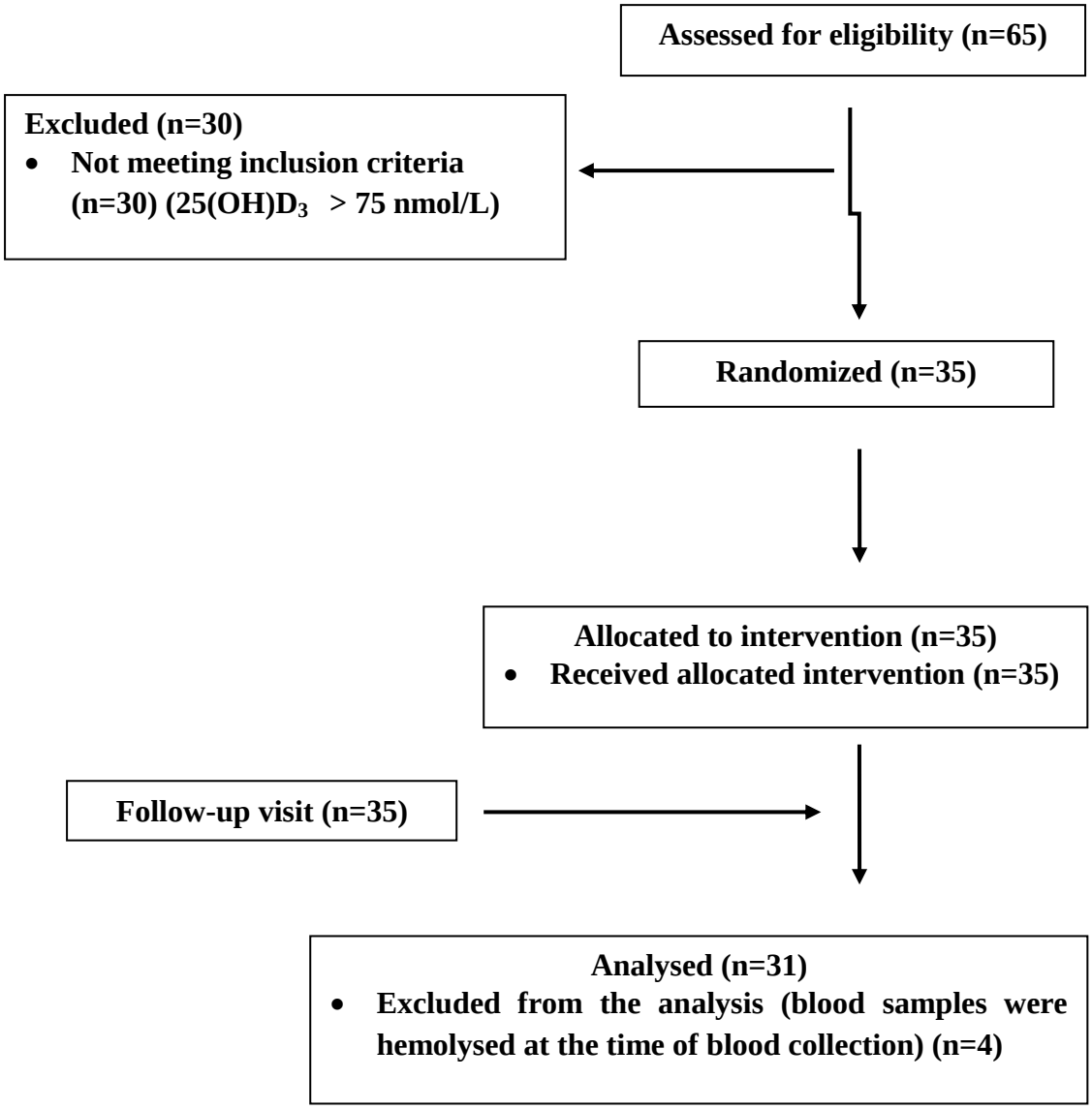

Figure 4.2 Consort flow diagram

4.4.2 Biochemical and physiological measures

A detailed medical history was examined for all subjects. Fasting blood was collected for the same parameters as part 1 of study. Resting blood pressure (systolic and diastolic) were obtained at baseline and at the follow-up visit. All blood pressure measurements were taken by certified research nurse using standardized procedures. Three blood pressure measurements were taken 5 minutes apart and average of the three measurements were used for the analyses.

4.4.3 Results of the part 2 of the study (study cohort 2)

4.4.3.1 Evaluation of the effects of vitamin D_3 supplementation in healthy volunteers (Study cohort 2)

Subject characteristics and biochemical profiles at baseline and follow up visits are shown in Chapter 3, Table 3.2 and Table 3.5. Of note, subjects in study cohort 2 had significantly lower 25(OH)D_3 and higher TSP-1 levels compared to cohort 1. Age, BMI and other metabolic parameters were comparable between the 2 study cohorts (data not shown).

Baseline platelet nitric oxide responsiveness (measured by percentage inhibition of ADP-induced platelet aggregation by SNP) was not correlated with baseline 25(OH)D_3 levels (β=-0.100, p=0.57) or PTH levels (β=-0.056, p=0.77). However, baseline platelet nitric oxide responsiveness showed a borderline inverse association with systolic blood pressure (β = -0.328, p = 0.06) and significant inverse association with diastolic blood pressure (β = -0.476, p = 0.01) levels.

4.4.3.1.1 Thrombospondin-1 levels decreased significantly after the treatment with vitamin D_3

Vitamin D supplementation for 12 weeks markedly reduced TSP-1 levels by almost 2.5 fold (592 ± 459 ng/mL, baseline vs 207 ± 204 ng/mL, follow-up; p<0.001) (Figure 4.3). However, there was no significant change in plasma ADMA concentrations (0.56±0.09 µmol/L, baseline vs 0.57±0.08 µmol/L, follow-up; p=0.59), ADP-induced platelet aggregation (8.2 ± 1.9 Ω, baseline vs 8.5 ± 2.2 Ω, follow-up; p=0.46) and platelet NO responsiveness (26.6±23.0

%, baseline vs 23.7±24.3 %, follow-up; p=0.12) after 12-weeks of vitamin D supplementation.

Changes in TSP-1 levels were not correlated with an improvement in vitamin D levels (β = 0.098, p = 0.57) in this study cohort. Furthermore, changes of TSP-1 levels were not significantly correlated with changes in PTH concentrations (β = -0.184, p = 0.69), fasting glucose levels (β = 0.350, p = 0.44), hs-CRP levels (β = -0.094, p = 0.84), ADMA levels (β = 0.133, p = 0.78), ADP-induced platelet aggregation (β = -0.005, p = 0.99) or platelet responsiveness to exogenous NO.

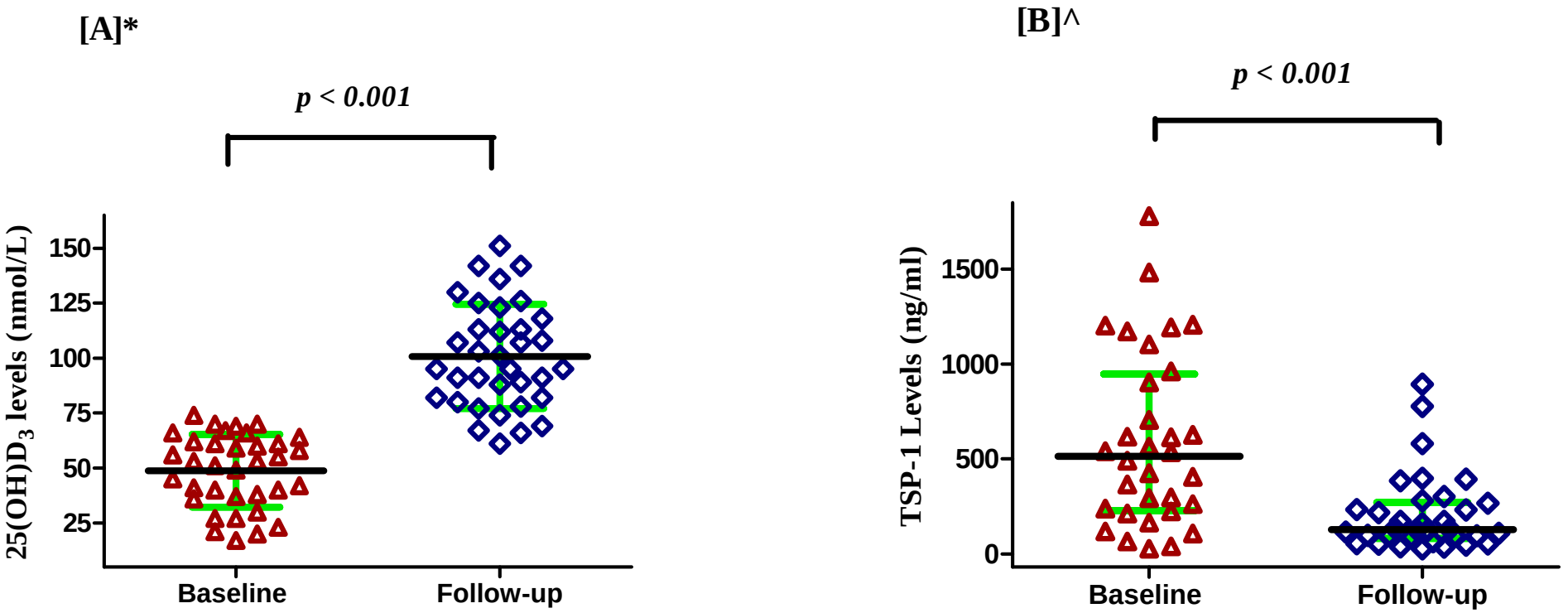

Figure 4.3 Significance changes of vitamin D levels and TSP-1 levels with vitamin D_3 replacement therapy in healthy volunteers (study cohort 2)

* Indicates mean±SD ^Indicates median (interquartile range)

4.5 Discussion

This study establishes that (i) there is an inverse relationship between plasma concentrations of vitamin D and TSP-1, and (ii) that increasing vitamin D concentrations in deficient individuals markedly decreases their TSP-1 concentrations. Therefore, these results strongly suggest that vitamin D exerts a major influence over TSP-1 release into plasma.

Previous research has reported the connection between vitamin D and cardiovascular homeostasis (Lee, J.H. et al. 2008; Temmerman 2011; Wang et al. 2012; Wang, T.J. et al. 2008), and similarly, that low vitamin D levels are reported to cause various coronary artery diseases (Szeto et al. 2012; Zittermann, A. & Koerfer 2008), and is an independent risk factor for cardiovascular events in general (Wang, T. J. et al. 2008) and for myocardial infarction independent of conventional cardiovascular risk factors (Giovannucci et al. 2008b); as well as for all-cause and cardiovascular mortality (Dobnig, Harald et al. 2008; Dobnig, H. et al. 2008a; Naesgaard et al. 2012; Schierbeck et al. 2011; Tomson, J. et al. 2013; Zhao et al. 2012). While the importance of vitamin D in cardiovascular disease/outcomes is apparent, mechanistic insights of these findings remain largely elusive.

Increased oxidative stress and impaired vascular NO function have been suggested to be partly responsible for the adverse cardiovascular outcomes associated with low vitamin D in both animal and human studies. Vitamin D has been shown to increase activity and expression of eNOS, the enzyme critical to the generation and bioavailability of NO. For example, treatment of endothelial cells with calcitriol significantly reversed advanced glycation end product-induced down-regulation of eNOS mRNA and activity (Talmor et al. 2008b). Aortic eNOS expression and urinary nitrate/nitrite excretion were reduced in vitamin D receptor knockout mice (Aihara et al. 2004). In spontaneously hypertensive rats, authors found that chronic treatment with active vitamin D metabolite reduced endothelium-dependent vascular contractions in the aorta, resulting in a reduction in blood pressure and basal endothelial reactive oxygen species (Wong et al. 2010). Clinically, significant improvement in endothelial function, measured by flow mediated dilatation, after vitamin D_2 replacement were observed in patients with Type 2 diabetes (Sugden et al. 2008) and in healthy overweight African American adults (Harris et al. 2011).

TSP-1 is a large matricellular glycoprotein, first identified as a protein released from α-granules of thrombin-stimulated platelets upon tissue injury (Adams 1997). TSP-1 regulates multiple cellular events involved in tissue repair including hemostasis, cell adhesion, migration, and proliferation (reviewed by (Adams & Lawler 2011)). It has been shown that TSP-1 inhibits NO-stimulated activation of sGC in endothelial cells, vascular smooth muscle cells, platelets, and T cells (reviewed by (Roberts, D. D. et al. 2012)), with subsequent inactivation of cGMP-dependent protein kinases (Isenberg, J. S., Frazier & Roberts 2008). In addition to inhibiting NO/sGC signaling, TSP-1 can also inhibit NO production via disruption of the vascular endothelial growth factor pathway. Acetylcholine-mediated activation of NO production in endothelial cells is also inhibited by TSP1 via CD47 in a calcium-dependent manner (Bauer, EM et al. 2010). Therefore, it would be expected that decreased TSP-1levels in plasma might induce increased vasodilator tone (via NO/PGI2 signaling cascades) and indeed both systolic and diastolic blood pressure fell in parallel with a fall in plasma TSP-1 concentrations.

In addition, high plasma concentrations of TSP-1 have been found in patients with coronary artery disease and diabetes mellitus (Choi et al. 2012). Patients with arteriosclerosis such as those with peripheral artery disease have also been found to have elevated plasma TSP-1 concentrations (Smadja DM, ATVB 2011). Thus, elevated TSP-1 concentrations may be a biomarker of cardiovascular disease states.

Currently, there are limited data that describe the association between TSP-1 with vitamin D. In human aortic smooth muscle cells, the addition of vitamin D analogs calcitriol and paricalcitol led to a marked down-regulation of TSP-1 mRNA and protein expression. In addition to inhibition of NO signaling, TSP-1 has been shown to activate transforming-growth factor (TGF-β1). Conversely, 1,25-dihydroxyvitamin D, has been shown to completely abolish TGF-β1 -induced increase in TSP-1 in rat renal interstitial cells, with subsequent protection against renal interstitial myofibroblast activation (Yingjian et al. 2005). It is therefore entirely possible that the effects of vitamin D in suppressing TSP-1 could have the dual beneficial effects of: 1) improving vascular NO signaling, and 2) prevention of TSP-1 activation of TGF-β1-mediated pro-inflammatory/pro-fibrotic signaling.

Little is known about the regulation of TSP-1 concentrations in plasma. Theoretically, these should be controlled by two major processes: (a) rates of platelet degranulation, and (b) rates

of TSP-1 clearance from plasma. If vitamin D deficiency indeed leads to elevated plasma concentrations of TSP-1, one possibility is that vitamin D modulates platelet activation.

The other relevant issue is whether released TSP-1 is in equilibrium with TSP-1 concentrations/effects within platelets. TSP-1 inhibits both vascular and platelet sGC activity (Isenberg, Jeff S et al. 2009; Isenberg, Jeff S. et al. 2006; Isenberg, Jeff S et al. 2008; Isenberg, Jeff S., Wink & Roberts 2006) and therefore it might have been expected that as plasma TSP-1 fell with vitamin D supplementation, SNP response might have increased. However, no such trend was observed. Indeed, our previous investigations (Ngo et al. 2011) showed no relationship between plasma TSP-1 concentrations and SNP effect, and this lack of correlation existed at baseline in the current intervention cohort. One possible explanation for this is heterogeneity of intra-platelet TSP-1 concentrations, with lower concentrations existing outside α-granules.

This is a pilot study not designed to measure cardiovascular outcomes, but aimed towards investigating the relationship between vitamin D concentrations/effects and TSP-1 concentrations. The main limitation of this study remains in the ability to demonstrate cause-and-effect relationships conclusively in humans: at present such investigations can only be performed in cell culture or in small animal models. Nevertheless, the observed vitamin D effect on plasma TSP-1 levels was marked and robust and likely to be physiologically relevant.

In conclusion, the current investigations represent the first demonstration in humans that any mainstream therapy can reduce TSP-1 levels in vivo. This is of particular interest given the apparent role of TSP-1 as a marker and mediator of endothelial dysfunction, enhanced fibrosis and thrombus formation, matrix remodeling, apoptosis and angiogenesis (reviewed by Krishna and Colledge, 2013). We now describe that such a simple, safe, cheap and commonly used therapy as vitamin D supplementation can reduce TSP-1 levels and consequently may have beneficial effects on cardiovascular health. Given the widespread prevalence of vitamin D deficiency and its association with adverse cardiovascular events/disease states, current results provide a basis for further mechanistic basic science and clinical trial investigations into the regulation of TSP-1 by vitamin D and its potential therapeutic implications, including the development of specific TSP-1 antagonists, especially in vitamin D deficient subjects.

Chapter 5 Summary, conclusions and future perspectives

Vitamin D deficiency and secondary hyperparathyroidism, either in combination or independently, have been associated with increased cardiovascular (CV) events. However, the exact mechanisms underlying the putative detrimental effects of vitamin D and parathyroid hormone on the cardiovascular system are unclear. This thesis is the natural extension of previous findings regarding the association of low vitamin D levels and impaired nitric oxide homeostasis in an ageing population, giving rise to the possibility that the commonly associated vitamin D deficiency in obese diabetic patients could incrementally increase their risk of adverse cardiovascular outcomes by worsening inflammation and endothelial function. We have also extended this observation to examine whether this relationship is independent of parathyroid hormone. In obese diabetic patients, we found that the previously seen relationship between low vitamin D levels and increased plasma ADMA concentrations was not present, but rather there was a robust relationship between high parathyroid hormone and high ADMA levels, independent of vitamin D and other concomitant cardiovascular risk factors. Indeed, these findings, in no way have proven a "cause-and-effect" relationship between parathyroid hormone and nitric oxide homeostasis. Therefore, we proceeded to an intervention study to examine whether vitamin D supplementation, with subsequent concomitant change in parathyroid hormones in healthy, but otherwise vitamin D deficient adults would result in changes in ADMA concentrations. To our surprise, improvement in vitamin D levels with supplementation did not lower ADMA concentrations, but rather the change in parathyroid hormone as a result of vitamin D supplementation correlated significantly with the change in ADMA levels after 12 weeks of supplementation. Although, these results did not completely demonstrate a cause-and-effect relationship, we demonstrated that manipulation of parathyroid hormone could affect ADMA levels independent of vitamin D. To fully elucidate whether parathyroid hormone has a regulatory role towards ADMA, or vice versa, cell culture or animal models must be implemented. A reduction of parathyroid hormone, either by receptor inhibition or reduction of its synthesis in endothelial cells could be used to assess the reciprocal effects on nitric oxide homeostasis by ADMA. Conversely, an increase in ADMA levels by inhibition of its clearance receptor DDAH could be employed to examine parathyroid hormone homeostasis.

Given the fact that there are close inter-relationships between regulatory mechanisms of vitamin D, parathyroid hormone, and calcium bioavailability, it would be difficult to demonstrate the nexus between each individual factor and ADMA regulation.

In addition to ADMA, we sought to examine whether, another regulator of nitric oxide bioavailability and signaling, thrombospondin-1 is associated with vitamin D. Thrombospondin-1 is released largely from platelet alpha-granules, though endothelial cells and smooth muscle cells have been found to also release TSP-1 (see introduction section 1.2.5.2.5). Thrombospondin-1 is a potent inhibitor of nitric oxide signaling by inhibition of sGC and cGMP formation. Elevated plasma TSP-1 levels have been found to be correlated with vaso-occlusive crises and peripheral vascular disease; tissue TSP-1 protein and mRNA levels have been found to be over-expressed in the leg muscles of patients with ischemic peripheral vascular disease, and single nucleotide polymorphisms of TSP-1 are associated with higher rates of early myocardial infarction (Rogers et al. 2014). Taken together, TSP-1 is a potential biomarker for adverse cardiovascular outcomes.

Vitamin D has been suggested to be a regulator of TSP-1 in human vascular smooth muscle cells. The addition of vitamin D analogs markedly suppressed TSP-1 mRNA levels (introductory chapter 1, section 1.2.5.2.5, page 30). Although, to date no study has demonstrated the relationship between vitamin D and TSP-1 in animals or in humans, therefore, we sought to examine the relationship between vitamin D and TSP-1 in humans via two studies: 1) an associative study , and 2) an intervention study.

In the associative study, we examined the relationship between plasma vitamin D and TSP-1 levels in healthy adults without concomitant cardiovascular diseases and varying BMI, age and vitamin D levels. We found a robust relationship between plasma vitamin D and TSP-1 levels in this cohort, independent of BMI and age. We proceeded to assess whether vitamin D supplementation in healthy adults with vitamin D deficiency would lower TSP-1 levels. We found that vitamin D supplementation markedly reduced TSP-1 levels by almost 2-fold. These results demonstrated clearly that vitamin D could regulate TSP-1 concentrations and therefore, TSP-1 is a target for vitamin D. We also found no relationship between changes in

parathyroid hormone and TSP-1, indicating that this is mainly a vitamin D effect. In these pilot studies, we have demonstrated that there is regulatory nexus between vitamin D and TSP-1, though a cross-over, randomized, placebo controlled study is required to definitively assess this relationship. Assessment of cGMP formation and sGC activity should also be performed on blood cells such as platelets to determine whether the effects of vitamin D on nitric oxide signaling is via TSP-1. Recently, TSP-1 has been found to play a role in bone homeostasis with roles in maintaining bone matrix integrity and regulating osteoclast formation (Amend et al. 2014). It would be worthwhile investigating whether this newly found effect of TSP-1 on bone could also be via vitamin D.

There were several other findings relating to the effects of vitamin D. Supplementation of vitamin D resulted in a marked reduction of both systolic and diastolic blood pressure in normotensive adults. These results supported the role of vitamin D as a negative regulator of renin synthesis, and thus inhibitor of angiotensin II formation. These results should be interpreted with caution as we did not design the study to assess blood pressure effects, and we did not measure oxidative stress or angiotensin levels, therefore, we could only speculate that vitamin D supplementation lowers blood pressure via reduction of angiotensin levels. There was also some reduction of fasting glucose and insulin levels, though this was not statistically significant with the vitamin D supplementation in this study. Once again, the study was not powered to examine the glucose/insulin effect of vitamin D, and that randomized, placebo control study, with a cross-over design to adjust for seasonal changes should be performed to fully elucidate the effects of vitamin D on these various factors. Results from clinical trials with pre-defined cardiovascular end-points such as myocardial infarction, stroke and cardiovascular mortality are pending. These studies will prove whether vitamin D supplementation will have cardiovascular benefits in both healthy subjects and patients with low vitamin D levels.

Conclusion

In conclusion, these studies conducted both in healthy subjects and obese diabetic patients confirmed that low vitamin D levels and high PTH levels could be cardiovascular risk factors and correction of low vitamin D levels could minimize the cardiovascular risk from altered vitamin D levels and PTH levels. Further studies should evaluate the underlying mechanisms of vitamin D and PTH on ADMA and TSP-1 metabolism. Furthermore, results from clinical trials with pre-defined cardiovascular endpoints such as myocardial infarction, stroke and cardiovascular mortality are pending. These studies will prove whether vitamin D supplementation will have cardiovascular benefits in both healthy subjects and patients with low vitamin D levels.

Appendix A Research work carried out by the candidate

Research work and experiments performed by Ms Anjalee Thanuja Amarasekera

- Interviewing the study subjects for obese-diabetic study (Chapter 2) and enrolling them to the study at the Cardiology Outpatient Clinics.
- Enrolling eligible subjects for vitamin D intervention study (Chapter 3 and 4).
- Obtaining the informed consent from the eligible participants.
- Processing blood samples of the participants and appropriately storing those samples.
- Measurement of asymmetric dimethylaarginine (ADMA) levels of obese-diabetic subjects as well as healthy volunteers recruited for the studies.
- Measurement of nitric oxide responsiveness of the platelets obtained from healthy working adults for the intervention study.
- Measurement of thrombospondin I (TSP-1) levels of the obese-diabetic subjects and healthy working adults recruited for the studies.
- Collecting research data through interviews with patients / healthy adults, patient's medical records, medical database and laboratory experiments.
- Entering the data and maintaining the study database.
- Statistical data analysis of the collected data by using statistical packages such as SPSS, GraphPad Prism, Excel etc.

Appendix B Vitamin D_3 analysing method

Assessment of vitamin D_3 ($25(OH)D_3$) levels in the obese-diabetic cohort (Chapter 2)

Vitamin D_3 ($25(OH)D_3$) levels of the study were measured by SA Pathology.

Vitamin D_3 levels of seventy six (76) obese diabetic patients were measured by immunodiagnostic system-enzyme immunoassay (IDS-EIA) method (Biokit, Barcelona, Spain). Testing was performed according to the manufacturer's protocols.

The rest of samples (n=88) were assessed for vitamin D_3 levels by immunodiagnostic systems (IDS) automated competitive binding chemiluminescence 25-OHD (IDS-iSYS 25-OHD) assay (IDS, Boldon, UK) as per the manufacturer's instruction.

Both assay systems were validated at the SA Pathology laboratory and quality-control was carried out using the manufacturers' control sets in conjunction with the in-house quality controls. There was a strong agreement between IDS-iSYS 25-OHD assay and IDS-EIA assay for detecting $25(OH)D_3$ levels ($R^2 = 0.86$ Passing and Bablok fit) (Cluse et al. 2012).

Assessment of vitamin D_3 ($25(OH)D_3$) levels of volunteer subjects (Chapter 3 and 4)

Vitamin D_3 ($25(OH)D_3$) levels of the study were measured by SA Pathology.

The $25(OH)D_3$ levels of healthy volunteers (Chapter 3 and 4) were detected by liquid chromatography-mass spectroscopic (LC-MS/MS) method. The method has coefficient of variance (CV%) of 5.9% and recovery rate of 99 – 104% for 25(OH)D. Patient data comparison between the IDS-iSYS 25-OHD and LC-MS/MS methods demonstrated a strong relationship, with a Passing-Bablok fit of IDS-iSYS 25-OHD = 1.03 LC-MS/MS -6.53, R^2 = 0.82 (Cluse et al. 2012).

Appendix C Parathyroid hormone (intact PTH) analysing method

PTH

cobas®

Parathyroid hormone (parathormone, parathyrin) - PTH, intact

REF	Σ	SYSTEM
11972103 122	100	Elecsys 2010 MODULAR ANALYTICS E170 **cobas e** 411 **cobas e** 601 **cobas e** 602

English

Intended use

Immunoassay for the in vitro quantitative determination of intact parathyroid hormone in human serum and plasma for the differential diagnosis of hypercalcemia and hypocalcemia. This assay can be used intraoperatively.

The **e**lectro**c**hemi**l**uminescence **i**mmuno**a**ssay "ECLIA" is intended for use on Elecsys and **cobas e** immunoassay analyzers.

Summary

Parathyroid hormone (PTH) is formed in the parathyroid glands and secreted into the blood stream. Intact PTH consists of a single polypeptide chain containing 84 amino acids and has a molecular weight of approx. 9500 daltons.

The biologically active N-terminal fragment has a half-life of only a few minutes. Selective measurement of the (mainly) intact parathyroid hormone permits direct ascertainment of the secretory activity of the parathyroid glands.[1,2]

PTH, together with vitamin D and calcitonin, brings about mobilization of calcium and phosphate from the skeletal system and increases the uptake of calcium in the intestine and the excretion of phosphate via the kidneys. The constancy of the blood calcium level is ensured by the interaction of PTH and calcitonin. The secretion of PTH is inhibited by high calcium concentrations and promoted by low calcium concentrations.

Parathyroid gland disorders lead to elevated or depressed blood calcium levels (hypercalcemia or hypocalcemia) brought about by a change in the secretion of PTH.

Detection of subfunctioning parathyroid glands (hypoparathyroidism) requires the use of a highly sensitive test in order to be able to measure PTH levels well below normal.[3,4]

Hyperfunctioning of the parathyroid glands results in an increased secretion of PTH (hyperparathyroidism). Primary causes are adenomas of the parathyroid glands. In secondary hyperparathyroidism the blood calcium level is low as a result of other pathological states (e.g. vitamin D deficiency).

Today, great significance is attached to the determination of the PTH and calcium concentrations when assessing hyperparathyroidism.

The determination of PTH intraoperatively during adenoma resection in the parathyroid glands has also been reported for primary hyperparathyroidism,[5,6,7] secondary hyperparathyroidism relating to renal failure,[8,9] and tertiary hyperparathyroidism post renal transplant surgery.[10] Because PTH has a reported half life of 3-5 minutes,[11] a significant drop in PTH levels after resection of the abnormal gland or glands enables the surgeon to assess the completeness of resection and whether all hyperfunctioning parathyroid tissue has been removed from the patient.[12]

The NACB guidelines recommend that baseline samples be obtained preoperation and pre-excision of the suspected hyperfunctioning gland.[13] Specimens for PTH testing should be drawn at 5 and 10 minutes post resection and that a > 50 % reduction in PTH levels from the highest baseline be used as criteria for surgical success. Additional samples may be necessary as it has been shown that sensitivity can increase with time.[14] Failure of PTH to drop below recommended levels indicates that either 1) residual hyperfunctioning tissue is still present and further exploration may be necessary, as was in the case of two patients, both with a fifth ectopic parathyroid gland requiring further surgery,[7] or 2) a spike in PTH levels during adenoma mobilization occurred.[15] Intraoperative PTH measurements offer fast, reliable assessment when all hyperfunctioning parathyroid tissue has been removed during the surgical process.

The Elecsys assay for determining intact PTH employs a sandwich test principle in which a biotinylated monoclonal antibody reacts with the N-terminal fragment (1-37) and a monoclonal antibody labeled with a ruthenium complex[a)] reacts with the C-terminal fragment (38-84).

The antibodies used in this assay are reactive with epitopes in the amino acid regions 26-32 and 37-42.

a) Tris(2,2'-bipyridyl)ruthenium(II)-complex ($Ru(bpy)_3^{2+}$)

Test principle

Sandwich principle. Total duration of assay: 18 minutes.

- 1st incubation: 50 µL of sample, a biotinylated monoclonal PTH-specific antibody, and monoclonal PTH-specific antibody labeled with a ruthenium complex form a sandwich complex.
- 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Reagents - working solutions

The reagent rackpack is labeled as PTH.

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL:
Streptavidin-coated microparticles 0.72 mg/mL; preservative.

R1 Anti-PTH-Ab~biotin (gray cap), 1 bottle, 7 mL:
Biotinylated monoclonal anti-PTH antibody (mouse) 2.3 mg/L; phosphate buffer 100 mmol/L, pH 7.0; preservative.

R2 Anti-PTH-Ab~$Ru(bpy)_3^{2+}$ (black cap), 1 bottle, 7 mL:
Monoclonal anti-PTH antibody (mouse) labeled with ruthenium complex 2.0 mg/L; phosphate buffer 100 mmol/L, pH 7.0; preservative.

Precautions and warnings

For in vitro diagnostic use.

Exercise the normal precautions required for handling all laboratory reagents.

Disposal of all waste material should be in accordance with local guidelines.

Safety data sheet available for professional user on request.

Avoid foam formation in all reagents and sample types (specimens, calibrators and controls).

Reagent handling

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated.

All information required for correct operation is read in from the respective reagent barcodes.

Storage and stability

Store at 2-8 °C.

Do not freeze.

Store the Elecsys reagent kit **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Stability:	
unopened at 2-8 °C	up to the stated expiration date
after opening at 2-8 °C	12 weeks
on the analyzers	8 weeks

Specimen collection and preparation

Only the specimens listed below were tested and found acceptable.

Serum collected using standard sampling tubes.

K_3-EDTA plasma.

Because of the short half-life of PTH, it is recommended that, when serum is needed, the blood should be centrifuged immediately.

Preference should be given to K_3-EDTA plasma, as it is stable longer than serum.

Criterion: Method comparison serum versus plasma, slope 0.9-1.1 + intercept within < ± 2 x analytical sensitivity (LDL) + coefficient of correlation > 0.95.

Serum: Stable for 8 hours at 15-25 °C, 2 days at 2-8 °C, 6 months at -20 °C.

Plasma: Stable for 2 days at 15-25 °C, 3 days at 2-8 °C, 6 months at -20 °C.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Do not use samples and controls stabilized with azide.

Ensure the samples, calibrators and controls are at 20-25 °C prior to measurement.

Due to possible evaporation effects, samples, calibrators and controls on the analyzers should be analyzed/measured within 2 hours.

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- REF 11972219122, PTH CalSet, for 4 x 1 mL
- REF 05618860190, PreciControl Varia, for 2 x 3 mL each of PreciControl Varia 1 and 2
- General laboratory equipment
- Elecsys 2010, MODULAR ANALYTICS E170 or **cobas e** analyzer

Accessories for Elecsys 2010 and **cobas e** 411 analyzers:

- REF 11662988122, ProCell, 6 x 380 mL system buffer
- REF 11662970122, CleanCell, 6 x 380 mL measuring cell cleaning solution
- REF 11930346122, Elecsys SysWash, 1 x 500 mL washwater additive
- REF 11933159001, Adapter for SysClean
- REF 11706802001, Elecsys 2010 AssayCup, 60 x 60 reaction vessels
- REF 11706799001, Elecsys 2010 AssayTip, 30 x 120 pipette tips

Accessories for MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers:

- REF 04880340190, ProCell M, 2 x 2 L system buffer
- REF 04880293190, CleanCell M, 2 x 2 L measuring cell cleaning solution
- REF 03023141001, PC/CC-Cups, 12 cups to prewarm ProCell M and CleanCell M before use
- REF 03005712190, ProbeWash M, 12 x 70 mL cleaning solution for run finalization and rinsing during reagent change
- REF 03004899190, PreClean M, 5 x 600 mL detection cleaning solution
- REF 12102137001, AssayTip/AssayCup Combimagazine M, 48 magazines x 84 reaction vessels or pipette tips, waste bags
- REF 03023150001, WasteLiner, waste bags
- REF 03027651001, SysClean Adapter M

Accessories for all analyzers:

- REF 11298500316, Elecsys SysClean, 5 x 100 mL system cleaning solution

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

Resuspension of the microparticles takes place automatically prior to use. Read in the test-specific parameters via the reagent barcode. If in exceptional cases the barcode cannot be read, enter the 15-digit sequence of numbers.

MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers: PreClean M solution is necessary.

Bring the cooled reagents to approx. 20 °C and place on the reagent disk (20 °C) of the analyzer. Avoid foam formation. The system automatically regulates the temperature of the reagents and the opening/closing of the bottles.

Calibration

Traceability: This method has been standardized against a commercial PTH test (RIA). The recovery of the NIBSC 95/646 (WHO) standard was assessed by testing dilutions in human serum covering the measuring range (40-4000 pg/mL) on 16 analyzers (**cobas e** 411 and **cobas e** 601 analyzers). The mean recovery was 100 % ± 4 %.

Every Elecsys reagent set has a barcoded label containing specific information for calibration of the particular reagent lot. The predefined master curve is adapted to the analyzer using the relevant CalSet.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the reagent kit was registered on the analyzer). Renewed calibration is recommended as follows:

- after 12 weeks when using the same reagent lot
- after 7 days (when using the same reagent kit on the analyzer)
- as required: e.g. quality control findings outside the defined limits

Quality control

For quality control, use PreciControl Varia.

In addition, other suitable control material can be used.

Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per reagent kit, and following each calibration.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in pg/mL or pmol/L).

Conversion factors: pg/mL x 0.106 = pmol/L
pmol/L x 9.43 = pg/mL

Limitations - interference

The assay is unaffected by icterus (bilirubin < 1112 µmol/L or < 65 mg/dL), lipemia (Intralipid < 1500 mg/dL) and biotin (< 205 nmol/L or < 50 ng/mL).

The assay is affected by hemolysis ≥ 0.15 g/dL. Do not analyze samples that show visible signs of hemolysis.

Criterion: Recovery within ± 10 % of initial value.

Samples should not be taken from patients receiving therapy with high biotin doses (i.e. > 5 mg/day) until at least 8 hours following the last biotin administration.

No interference was observed from rheumatoid factors up to a concentration of 1500 IU/mL.

There is no high-dose hook effect at PTH concentrations up to 17000 pg/mL (1802 pmol/L).

In vitro tests were performed on 16 commonly used pharmaceuticals. No interference with the assay was found.

In rare cases, interference due to extremely high titers of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur. These effects are minimized by suitable test design.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Limits and ranges

Measuring range

1.20-5000 pg/mL or 0.127-530 pmol/L (defined by the lower detection limit and the maximum of the master curve). Values below the lower detection limit are reported as < 1.20 pg/mL (< 0.127 pmol/L). Values above the measuring range are reported as > 5000 pg/mL (> 530 pmol/L).

Lower limits of measurement

Lower detection limit of the test

Lower detection limit: 1.20 pg/mL (0.127 pmol/L)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying two standard deviations above that of the lowest standard (master calibrator, standard 1 + 2 SD, repeatability study, n = 21).

Dilution

Not necessary due to the broad measuring range.

Expected values

15-65 pg/mL (1.6-6.9 pmol/L)[16,17]

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using Elecsys reagents and pooled human sera in a modified protocol (EP5-A) of the CLSI (Clinical and Laboratory Standards Institute): 6 times daily for 10 days (n = 60); repeatability on MODULAR ANALYTICS E170 analyzer, n = 21. The following results were obtained:

Elecsys 2010 and **cobas e** 411 analyzers								
			Repeatability			**Intermediate precision**		
Sample	Mean		SD		CV	SD		CV
	pg/mL	pmol/L	pg/mL	pmol/L	%	pg/mL	pmol/L	%
HS[b)] 1	26.7	2.83	0.711	0.075	2.7	1.73	0.184	6.5
HS 2	52.5	5.56	0.853	0.091	1.6	2.07	0.220	3.9
HS 3	261	27.7	4.0	0.424	1.5	7.81	0.829	3.0

b) HS = human serum

MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers					
	Repeatability				
Sample	Mean		SD		CV
	pg/mL	pmol/L	pg/mL	pmol/L	%
HS 1	21.9	2.32	0.44	0.05	2.0
HS 2	35.0	3.71	0.43	0.05	1.2
HS 3	123	13.04	1.31	0.14	1.1

MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers					
	Intermediate precision				
Sample	Mean		SD		CV
	pg/mL	pmol/L	pg/mL	pmol/L	%
HS 1	23.2	2.46	0.79	0.08	3.4
HS 2	80.9	8.58	2.01	0.21	2.5
HS 3	240	25.4	6.72	0.71	2.8

Precision was determined using Elecsys reagents and controls in a separate study according to protocol EP5-A2 of the CLSI (Clinical and Laboratory Standards Institute): 2 runs per day in duplication each for 21 days (n = 84). The following results were obtained:

Elecsys 2010 and **cobas e** 411 analyzers								
			Repeatability			**Intermediate precision**		
Sample	Mean		SD		CV	SD		CV
	pg/mL	pmol/L	pg/mL	pmol/L	%	pg/mL	pmol/L	%
PC[c)] Varia 1	54.6	5.79	0.657	0.070	1.2	1.11	0.118	2.0
PC Varia 2	182	19.3	2.43	0.258	1.3	3.14	0.333	1.7

c) PC = PreciControl

MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers								
			Repeatability			**Intermediate precision**		
Sample	Mean		SD		CV	SD		CV
	pg/mL	pmol/L	pg/mL	pmol/L	%	pg/mL	pmol/L	%
PC Varia 1	54.7	5.80	0.943	0.100	1.7	0.977	0.104	1.8
PC Varia 2	184	19.5	2.65	0.281	1.4	3.11	0.330	1.7

Method comparison

A comparison of the Elecsys PTH assay (y) with a commercial PTH test (x) using clinical samples gave the following correlations (pg/mL):

Number of samples measured: 152

Passing/Bablok[18]	Linear regression
y = 1.01x + 4.86	y = 0.83x + 19.4
τ = 0.886	r = 0.991

The sample concentrations were between approx. 1.4 and 1880 pg/mL (0.15 and 199 pmol/L).

Analytical specificity

No cross-reactivities were found for: Osteocalcin, PTH fragment 1-37, PTH-related protein (1-86), bone-specific alkaline phosphatase, and β-CrossLaps.

Functional sensitivity

6.0 pg/mL (0.64 pmol/L)

The functional sensitivity is the lowest analyte concentration that can be reproducibly measured with an intermediate precision CV of < 20 %.

Clinical investigations in intraoperative use

In 2006, the National Academy of Clinical Biochemistry published their Laboratory Medicine Practice Guidelines for point of care testing, entitled Evidence Based Practice for Point of Care Testing.[13] The guidelines recommend the use of intraoperative parathyroid hormone testing 1) for patients undergoing surgery for hyperparathyroidism, especially in minimally invasive or directed procedures, 2) for patients undergoing reoperation, and 3) as a replacement for traditional laboratory measurements of PTH during venous localization in order to help the angiography team guide sampling.

The guidelines further recommend for patients undergoing parathyroidectomy for hyperparathyroidism that baseline samples be obtained preoperation exploration and pre-excision of the gland, and that post-excision sampling be drawn at 5 and 10 minutes post resection with a 50 % reduction in PTH concentrations from the highest baseline level. The guidelines also caution that additional samples may be necessary.[13]

PTH testing during parathyroid surgery was conducted by several groups of investigators using the Elecsys PTH immunoassay.[6,7,8,9,10]

The overall sensitivity and specificity of the assay to demonstrate successful surgery as defined by postoperative reduction of calcium levels was 99.6 % and 93.7 %, respectively.

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For further information, please refer to the appropriate operator's manual for the analyzer concerned, the respective application sheets, the product information and the Method Sheets of all necessary components (if available in your country).

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard.

CONTENT	Contents of kit
SYSTEM	Analyzers/Instruments on which reagents can be used
REAGENT	Reagent
CALIBRATOR	Calibrator
→	Volume after reconstitution or mixing

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All other product names and trademarks are the property of their respective owners.

Significant additions or changes are indicated by a change bar in the margin.

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PTH

Parathyroid hormone (parathormone, parathyrin) - PTH, intact

CE

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim
www.roche.com

Appendix D Patient information sheet - Does vitamin D deficiency contribute to endothelial dysfunction in diabetes?

Government of South Australia
Central Northern Adelaide Health Service

CENTRAL NORTHERN ADELAIDE HEALTH SERVICE

The Queen Elizabeth Hospital & Lyell McEwin Hospital

Patient Information Sheet

Title: Does vitamin D deficiency contribute to endothelial dysfunction in diabetes?

Protocol Number: 2009029

INVITATION TO PARTICIPATE

We invite you to participate in a research project which we believe is of potential importance in identifying the significance of vitamin D deficiency in patients with obesity and diabetes. Vitamin D deficiency has been shown to poorly affect cardiovascular health, and we hope to investigate whether vitamin D supplementation will improve markers associated with cardiovascular health. We hope that the findings of this study will help us better manage this condition in future.

However, before you decide whether or not you wish to participate, we need to be sure that you understand

why we are doing it, and
what it would involve if you agreed.

We are therefore providing you with the following information.
Please read it carefully and be sure to ask any questions you have.
The Doctor conducting the research will be happy to discuss it with you and answer any questions that you may have.
You are also free to discuss it with outsiders if you wish. (Ie family, friends and / or your local Doctor)

You do not have to make an immediate decision.
Your participation is purely voluntary.
Should you agree to enter the trial, you may change your mind and withdraw at any stage.

PARTICIPATION IS VOLUNTARY
Participation in any research project is voluntary. If you do not wish to take part, you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage without providing a reason.

Your decision to take part, not to take part or to withdraw will not affect your routine treatment, your relationship with those treating you, or your relationship with The Queen Elizabeth Hospital.

Background to the study

We know that diabetic patients who are obese have a high incidence of vitamin D deficiency. Low vitamin D levels may be bad for cardiovascular health. This study will firstly identify the incidence of vitamin D deficiency in this group of patients, and then whether vitamin D supplementation will improve platelet and blood vessel function of these patients.

We will be screening 200 patients. We will be inviting a total of 40 patients with vitamin D deficiency to participate in an evaluation of the effects of correction of the deficiency with extra vitamin D.

This research study is funded by The Queen Elizabeth Hospital.

PROCEDURES AND TREATMENT

The study will take place at The Queen Elizabeth Hospital on 2 visits. Before the study can begin you will need to be assessed to see if you are eligible for entry into the study. You will be excluded from this study if you have previous allergies to vitamin D therapy, have severe asthma or previous adverse reactions to the listed medications (See below – Medicines or Drugs). You will then be asked to sign a consent form if you decide to be a participant in the study.

Study day:

You will be asked to attend the study for evaluation on 4 extra occasions at 3 monthly intervals, if you are vitamin D deficient.

Part 1 - Screening:

This will involve an interview about your health, and the taking of a single blood sample (total 20mls) as a general blood test as well as to check your vitamin D levels.

Part 2 – Intervention:

If you are identified as having vitamin D deficiency, you will be invited to participate in the intervention stage of the study. The following will be performed at each of the 4 assessment periods:

Fifteen minutes before this part of the study commence, you will be asked to rest in a lying down position. You will remain in this position for the duration of this part of the study (≈ 45 minutes).

The following will then be performed:

1) A blood sample (total volume 60mls) which will be withdrawn from a vein for the analysis of platelet function and endogenous (within the body) levels of substances which modulate platelet function. Platelets are small cells within the blood stream that are responsible for clotting. We will also measure levels of endothelial progenitor cells, which are the cells responsible for repairing damaged blood vessels.

2) An ultrasound of your upper arm's artery will be conducted. This will involve you lying down in a comfortable position for 15 to 20 minutes while the ultrasound is performed. The change in diameter of your upper arm artery will be assessed after release of a tight blood pressure cuff and drug administration (glyceryl trinitrate).

MEDICINES AND DRUGS

You will be given in the study:

1) A small dose of a drug (50 mcg) called glyceryl trinitrate (a low dose compared to that used in patients with angina/heart disease), given sublingually (under the tongue).
2) The study has 3 phases: each of 3 months' duration, separated by a 3 months' treatment (washout) phase. You will be required to take 3 capsules each morning: these might be vitamin D3 capsules or placebo for either the first or second treatment phase.
3) During the study period, we would request that you do not take additional tablets known to contain vitamin D.

PATIENT MANAGEMENT

You can withdraw from the study at anytime: however, it is most unlikely that there will be any side effects from this dose of vitamin D3.

DISCOMFORTS, RISKS AND SIDE EFFECTS

During part 2 of the study:
The blood sample will be taken via intravenous access (a needle placed in your arm): this causes mild discomfort and may lead to local bruising (risk of bruising < 10%).

The possible side effects of glyceryl trinitrate include headache, dizziness, flushing, fast heart rate, low blood pressure and fainting. However, the effects are very short-lived. (<10%)

This part will conclude only when your blood pressure has returned to normal.

Application of a tight blood pressure cuff around your forearm for 5 minutes during the ultrasound of your upper arm artery (brachial artery) may cause mild discomfort in your forearm. This will be short-lived.

Part 2 of the study

It is not anticipated that the dose of vitamin D supplementation in this study will be harmful. But if you do experience any discomfort that coincides with vitamin D supplementation, you should let the investigators know, and the study will be terminated.

PREGNANCY

Patients will be excluded if they are pregnant.

WHAT WILL HAPPEN TO THE INFORMATION COLLECTED?

Your participation in this study will be treated as confidential. Any records or results relating to the study shall not be disclosed to any third party other than your referring Cardiologist, representatives of The Ethics of Human Research Committee at the Central Northern Adelaide Health Service, or regulatory bodies for medicines, or as may otherwise be required by law. The data will be held locally for the duration of the study. Your identity will remain confidential. The Central Northern Adelaide Health Service Cardiology Unit will ensure that they comply with any national drug protection regulation.

Your usual doctor will be informed of your participation, and at the end of the study, of your vitamin D status.

WHAT ARE MY RIGHTS?

If you become injured during this study, and your injury is a direct result of the effects of study procedures, The Queen Elizabeth Hospital will provide reasonable medical treatment. Your participation in this study shall not affect any other right to compensation you may have under common law.

To obtain more information, please feel free to contact us (details provided below)

IS THERE ANY PAYMENT FOR PARTICIPATION?

There will be no payment for your participation in the study. There is no anticipated cost to you other than your time.

If you sustain a bodily injury that is caused by the study procedure, you will be reimbursed for reasonable physicians' fees and medical expenses necessary for treatment of the injury not covered by medical and hospital insurance.

BENEFITS OF THE RESEARCH

Participating in the study may benefit you directly by improving your vitamin D levels. However, it will help also us better understand the risks and outcomes of the patients with vitamin D deficiency, obesity and diabetes, and subsequently may affect our management of these patients**.**

WHAT IF I HAVE A QUESTION ABOUT THE STUDY?

You have the right to ask any questions concerning the potential and/or known hazards of this study at any time. You will be informed of any significant new information pertaining to your safety. If you have any questions concerning the study please contact:

Principal Investigator
Prof John Horowitz
Cardiology Unit
The Queen Elizabeth Hospital
Tel: 82226000 pager 20650 (at all times)

Or

Co-Investigator
Dr Doan Ngo
Cardiology Unit
The Queen Elizabeth Hospital
Tel: 82226000 pager 6678

Or

Co-Investigator
Dr Aaron Sverdlov
Cardiology Unit
The Queen Elizabeth Hospital
Tel: 82226000 pager 47947

The Central Northern Adelaide Health Service Ethics of Human Research Committee (TQEH & LMH) has approved this study.
Should you wish to speak to a person not directly involved in the study in relation to

- matters concerning policies,
- information about the conduct of the study
- your rights as a participant, or

should you wish to make a confidential complaint, you may contact The Executive Officer of this Committee, on (08) 8222 6841

Government of South Australia
Central Northern Adelaide Health Service

CENTRAL NORTHERN ADELAIDE HEALTH SERVICE

The Queen Elizabeth Hospital & Lyell McEwin Hospital

consent form

Title: Does vitamin D deficiency contribute to endothelial dysfunction in diabetes?

Protocol Number:2009029

I, the undersigned .....

hereby consent to my involvement in the research project explained above.

- I have read the information sheet, and I understand the reasons for this study. The research worker has explained the ways in which it will affect me. My questions have been answered to my satisfaction. My consent is given voluntarily.

- I understand that the purpose of this research project is to improve the quality of medical care, but my involvement may not be of benefit to me.

- The details of the research project have been explained to me, including:
  - The expected time it will take
  - The nature of any procedures being performed, and the number of times they will be performed
  - The nature of any medications I may be given
  - Any discomfort which I may experience

- I have been given the opportunity to have a member of family or a friend present while the project was explained to me.
- My identity will be kept confidential, and nothing will be published which could possibly reveal my identity.
- My involvement in the study will not affect my relationship with my medical advisers. I understand that I am able to withdraw from the study at any stage without having to give a reason, and that by withdrawing it will not affect my treatment at this hospital in the future.

PATIENT SIGNATURE ..... **DATE** ……./……./…….

WITNESS ..... **DATE** ……./……./…….

INVESTIGATOR ..... **DATE** ……./……./…….

Appendix E Patient information sheet - Does vitamin D replacement improve endothelial function in healthy adults?

Government of South Australia
SA Health

A Campus of Central Adelaide Local Health Network
The Queen Elizabeth Hospital
28 Woodville Road
Woodville South SA 5011
ABN 96 269 526 412
www.tqeh.sa.gov.au

CENTRAL ADELAIDE LOCAL HEALTH NETWORK
The Queen Elizabeth Hospital

PARTICIPANT INFORMATION SHEET

Title: Does vitamin D replacement improve endothelial function in healthy adults?
Protocol Number: HREC/12/TQEHLMH/20

INVITATION TO PARTICIPATE

We invite you to participate in a research project which we believe is of potential importance. However, before you decide whether or not you wish to participate, we need to be sure that you understand

why we are doing it, and
what it would involve if you agreed.

We are therefore providing you with the following information.
Please read it carefully and be sure to ask any questions you have.
The Doctor conducting the research will be happy to discuss it with you and answer any questions that you may have.
You are also free to discuss it with outsiders if you wish. (i.e. family, friends and / or your local Doctor)
You do not have to make an immediate decision.

PARTICIPATION IS VOLUNTARY

Participation in any research project is voluntary. If you do not wish to take part, you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage without providing a reason.

Your decision to take part, not to take part or to withdraw will not affect your routine treatment, your relationship with those treating you, or your relationship with The Queen Elizabeth Hospital.

BACKGROUND TO THE STUDY

What is the research about? Why is the research being done?
Recently, vitamin D deficiency has been suggested as bad for cardiovascular health. We have discovered that about 75% of healthy working volunteers are vitamin D deficient. In this study, we will assess whether vitamin D supplementation for 12 weeks will improve your blood vessel function.

This study will screen 100 healthy volunteers, of which 35 participants with low vitamin D levels will be invited to receive vitamin D supplementation for 12 weeks.

The research is being initiated/sponsored by the Cardiology Department at The Queen Elizabeth Hospital and is receiving no payment from outside sources.

How and why have I been chosen as a possible participant in the research?
You have been chosen because you are a healthy volunteer who may be vitamin D deficient.

How many other people have been asked to consider participating?
We are conducting this study at The Queen Elizabeth Hospital (TQEH) only. The first part of the study will involve screening a total of 100 participants, of which 35 with vitamin D deficiency will be invited for the second part of the study.

PROCEDURES AND TREATMENT

The study will take place at The Queen Elizabeth Hospital on 3 visits.

Part 1 Assessment (visit 1) - Before the study can begin you will be asked to sign a consent form. You will then be assessed to see if you are eligible for entry into the study. You will be excluded from this study if you are taking any medications for treatment of cardiovascular diseases, diabetes, have had any previous allergies to vitamin D therapy, or are pregnant or breastfeeding. If you do not have any exclusion criteria then a blood sample will be taken to assess your vitamin D levels.

Part 2: Intervention (visit 2) - If you are identified as having vitamin D deficiency, you will be invited to participate in the intervention stage of the study. At this visit a blood sample (total volume of 30mls, or 1½ tablespoons) will be withdrawn from a vein for the analysis of platelet function and endogenous (within the body) levels of substances which modulate platelet function. Platelets are small cells within the blood stream that are responsible for clotting. You will then be given a bottle of Vitamin D capsules, each capsule containing 2000IU of vitamin D. You will be asked to take 1 vitamin D capsule daily for a period of 12 weeks.

Visit 3 - At this visit after you have been taking the vitamin D supplement for 12 weeks you will again have blood sampling (about 30 ml) to test for the same substances in your blood as in visit 2. Any Vitamin D capsules that you have remaining, will be collected from you at this visit.

Additional procedures or treatment
There will be no additional procedures or exposures for you during this study beyond normal standard of care.
No additional drugs/treatments will be given other than Vitamin D capsules.
There will be no changes to your usual health treatment or decision-making processes and your normal doctor will continue managing your care.

Are there any factors, which would exclude me from participating, like pre-existing illness, the possibility of becoming pregnant or other drugs being taken?
As mentioned above you will not be able to participate if you are pregnant or have cardiovascular disease which requires medication or are known to have a vitamin D allergy. You are not allowed to take any other Vitamin D supplements whilst you are in the study.

MEDICINES AND DRUGS

You will be taking 1 vitamin D capsule, 2000IU daily. The capsules are prepared by Blackmore's. You will take the capsule orally.

PATIENT MANAGEMENT

What would happen if I were to feel severe discomfort or pain?

You are not expected to feel severe discomfort or pain during this study; however, you may feel some small discomfort during blood taking, if you should feel unwell; please alert the study staff at the time.

DISCOMFORTS, RISKS AND SIDE EFFECTS

Will there be any discomforts, such as additional needles, biopsies, or pain?
There are no additional discomforts other than those associated with blood taking. There is a very small risk of bruising or infection at the blood collection site.

Who should I contact if I am worried about any effects that I experience?
It is not expected that you would suffer any effects from the Vitamin D. However, you can contact any of the study personnel listed on this form or your usual treating doctor.

Are there any activities I should refrain from during and in the period following the research, and for how long?
It is not expected that you refrain from any normal activities. Pregnancy is an exclusion criteria for this study so if you happen to become pregnant during the study period, please notify the study staff so that a decision can be made if you are to remain in the study or not.

WHAT WILL HAPPEN TO THE INFORMATION COLLECTED?

Participant's information/results will be de-identified and you will be given a study number. Only study investigators will have access to the participant's identity information. You will not be identified in any publications arising from this work. The information collected can be made available to you and your doctor, but is essentially of scientific value only. All information will be stored in accordance with local and federal legislation. (15 years)

Additionally, when you have finished your participation in the study, both you and your General Practitioner (GP) will be given the final results of your Vitamin D levels. Any continuing treatment that is required with Vitamin D and subsequent blood tests will be managed by your GP.

WHAT ARE MY RIGHTS?

If you become injured during this study, and your injury is a direct result of the effects of study procedures, The Queen Elizabeth Hospital will provide reasonable medical treatment.

Your participation in this study shall not affect any other right to compensation you may have under common law.

IS THERE ANY PAYMENT FOR PARTICIPATION?

There is no financial reimbursement for participation in this study. However, we can provide taxi vouchers if required for your visits to the hospital.

BENEFITS OF THE RESEARCH

We cannot guarantee that there will be any benefits to you personally; however, this research may improve our understanding of mechanisms underlying vitamin D and endothelial function.

WHAT IF I HAVE A QUESTION ABOUT THE STUDY?

Please contact the following people if you require any further information about the study:

Principal Investigator
Professor John Horowitz
Cardiology Unit,
The Queen Elizabeth Hospital
Tel: 8222 6000, pager 47679

The Human Research Ethics Committee (TQEH/LMH/MH) has approved this study. Should you wish to speak to a person not directly involved in the study in relation to

- matters concerning policies,
- information about the conduct of the study
- your rights as a participant, or should you wish to make a confidential complaint,

you may contact The Executive Officer of this Committee, on (08) 8222 6841

Government of South Australia
SA Health

A Campus of Central Adelaide Local Health Network
The Queen Elizabeth Hospital
28 Woodville Road
Woodville South SA 5011
ABN 96 269 526 412
www.tqeh.sa.gov.au

CENTRAL ADELAIDE LOCAL HEALTH NETWORK
The Queen Elizabeth Hospital

CONSENT FORM

Title: Does vitamin D replacement improve endothelial function in healthy adults?
Protocol Number: HREC/12/TQEHLMH/20

I, the undersigned .....

hereby consent to my involvement in the research project explained above.

- I have read the information sheet, and I understand the reasons for this study. The research worker has explained the ways in which it will affect me. My questions have been answered to my satisfaction. My consent is given voluntarily.

- I understand that the purpose of this research project is to improve the quality of medical care, but my involvement may not be of benefit to me.

- The details of the research project have been explained to me, including:
  - The expected time it will take
  - The nature of any procedures being performed, and the number of times they will be performed
  - The nature of any medications I may be given
  - Any discomfort which I may experience

- I have been given the opportunity to have a member of family or a friend present while the project was explained to me.
- My identity will be kept confidential, and nothing will be published which could possibly reveal my identity.
- I understand that I should not be pregnant during the course of this trial. In the event of pregnancy occurring, I will notify the investigator as soon as practically possible.
- My involvement in the study will not affect my relationship with my medical advisers. I understand that I am able to withdraw from the study at any stage without having to give a reason, and that by withdrawing it will not affect my treatment at this hospital in the future.

PATIENT SIGNATURE..... **DATE** ……./……./…….

INVESTIGATOR..... **DATE** ……./……./…….

WITNESS: (if required) .....**DATE** ……./……./…….

Appendix F Enrolment visit form and Sun exposure questionnaire

Department of Cardiology Research - The Queen Elizabeth Hospital, Adelaide, SA

'Vitamin D in Healthy Volunteers'

ENROLMENT VISIT

MUST MEET ALL INC / EXC CRITERIA	➢ Vitamin D < 75 nmol/L? ➢ Over 18 years of age? ➢ No diagnoses of diabetes mellitus or CV disease? ➢ Provided informed consent? ➢ Haven't had previous reactions to Vitamin D supplements? ➢ Not pregnant or breastfeeding? ➢ Not employed by Cardiology?

DOCTOR	This patient has been consented to '*Vitamin D in Healthy Volunteers'*. He/she meets all the inclusion criteria and none of the exclusion criteria. The study has been explained by the study nurse and investigator, and the patient has willingly agreed to participate and has been consented by Dr……..……on……. at……hrs. Consent version…… dated……. was signed. A copy of the consent has been given to the patient and they were given time to review the consent and ask questions.
SIGN HERE >	**Doctors signature & date** ……**…**
TO DO >	➢ Review, sign and date initial Vitamin D result ➢ Ensure all inc / exc criteria is met ➢ Explain study to patient ➢ Sign consent form ➢ Complete Vitamin D script

VITAL SIGNS	➢ Height …….. cms ➢ Weight ……Kg	➢ BSA ……m2 ➢ BP ……/…….

SUN EXPOSURE QUESTIONS	➢ How many hours are worked per week? ➢ How many hours are spent indoors per week? ➢ What outdoor activities do they partake in?
NEXT APPOINTMENT	➢ Due in 12 weeks, booked for …….. ➢ Enter appointment into cardiology research calendar

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