Vitamin D, Endothelial Function and Cardio-Metabolic Health

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Abstract

The classical actions of vitamin D are to maintain bone health but vitamin D receptors are also expressed in several other tissues including the vascular system. The epidemiological evidence supports the associations between vitamin D status and vascular health but it is still uncertain whether the inadequate vitamin D status represents a risk factor for cardiovascular diseases (CVD). Nitric oxide (NO) is a soluble gas synthesized from the amino acid L-arginine and plays an important role in maintaining vascular homeostasis. A reduced NO availability is an important hallmark of endothelial dysfunction, which is closely linked to the development of atherosclerosis and is an independent risk factor for cardiovascular events. Vitamin D may be involved in the maintenance of endothelial function (EF) through regulation of endothelium nitric oxide synthase (eNOS) activity which enhanced NO synthesis. This PhD project investigated the association between vitamin D intake and status, endothelial function and biomarkers of NO availability. In the first phase of the PhD, a systematic review and meta-analysis of randomized clinical trials was conducted to investigating the effects of vitamin D supplementation on EF. Overall, this showed no improvement of EF after vitamin D supplementation but there was evidence of positive effects in obese individuals and in diabetic patients. Next, a cross sectional study in post-menopausal overweight and obese women was conducted to evaluate the associations between vitamin D status and physiological and circulating biomarkers of EF. The study revealed increased pulse wave velocity (PWV), a marker of arterial stiffness, and higher concentrations of asymmetric dimethylarginine (ADMA, a marker of NO availability) in those women with vitamin D concentrations < 30 nmol/L. Detailed laboratory experiments were also conducted as part of this project to improve the measurement of concentrations of NO_3^- and NO_2^- in plasma and urine using ozone-based chemiluminescence. The final phase of the project investigated the association between whole-body NO availability (measured by a non-invasive stable-isotopic method), PWV, ADMA and vitamin D status and whether these associations were modified by age. The study did not find a significant association of NO production with vitamin D. However, NO production was significantly lower in older individuals and was significantly associated with systolic blood pressure and PWV.

Supervisors' Certificate

This is to certify that the entitled thesis "**Vitamin D, Endothelial Function and Cardio-Metabolic Health**" has been prepared under my supervision at the Institute of Cellular Medicine/Newcastle University for the degree of PhD in Nutrition.

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Dedicated to:

My Beloved Parents

My Dearest Husband and Lovely Kids

My Supportive Family

&

My Wonderful Friends for Their Assistance

Thank You for Everything

'Life is like riding a bicycle. In order to maintain balance, we must keep moving'

Albert Einstein

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Publications

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Conferences Abstracts

Mat Hussin A, Ashor WA, Schoenmakers I, Hill T, Mathers CJ, Siervo S (2015). Effects of Vitamin D Supplementation On Endothelial Function: A Systematic Review and Meta-Analysis of Randomised Controlled Trials. 18th Workshop of Vitamin D, Delft, Netherlands.

Mat Hussin A, Ashor WA, Schoenmakers I, Hill T, Mathers CJ, Siervo S (2015). Effects of Vitamin D Supplementation On Endothelial Function: A Systematic Review and Meta-Analysis of Randomised Controlled Trials. Newcastle University Institute for Ageing (NUIA) Postgraduate Student Research Day, Great North Museum, Newcastle, UK.

Mat Hussin A, Ashor WA, Schoenmakers I, Hill T, Mathers CJ, Siervo S (2016). Association of Vitamin D with Physiological and Biochemical Markers of Endothelial Function in Overweight and Obese Post-Menopausal Women. Proceedings of The Nutrition Society, Dublin, Ireland.

Grants and Awards

- NUIA Travel Grant Award (23/05/2016)
- Best Poster for HNRC Day (26/10/2016)

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List of Abbreviations

A

- ADMA = Asymmetric Dimethylarginine
- AFRD = Agriculture, Food and Rural Development
- AH = Area of Hyperemia
- AI = Augmentation Index
- AngII = Angiotensin II
- ANP = Atrial Natriuretic Peptide
- AO = Area of Occlusion
- ARD = Analogue Recorder and Display
- AUC = Area Under Curve

B

- B & A = Bland Altman
- BH_4 = Tetrahydrobiopterin
- BMI = Body Mass Index
- BP = Blood Pressure

C

- Ca+ = Calcium Cation
- CAD = Coronary Artery Disease
- CARU = Clinical Ageing Research Unit
- CFS/ME = Chronic Fatigue Syndrome/ Myalgic Encephalomyelitis
- cGMP = Cyclic Guanosine Monophosphate
- CHD = Coronary Heart Disease
- CI = Confidence Interval
- CFS = Chronic Fatigue syndrome
- CKD = Chronic Kidney Disease
- CL = Chemiluminescence
- CLIA = Chemiluminescence Immunoassay
- CRESTA = Clinics for Research and Service in Themed Assessment
- CV = Coefficient of Variability
- CVD = Cardiovascular Disease
- CYP2R1 = Cytochrome P450 2R1

D

- D_2 = Ergocalciferol
- D_3 = Cholecalciferol
- DBP = Diastolic Blood Pressure
- DBS = Dry Blood Spot
- 7-DHC = 7-Dehydrocholesterol
- DC = Distensibility Coefficient
- DEQAS = Vitamin D External Quality Assurance Scheme
- DNA = Deoxyribonucleic Acid
- DRIs = Dietary References Intake

E

- ECG = Electrocardiogram
- EDD = Endothelium Dependent Dilation
- EDHF = Endothelium-Derived Hyperpolarizing Factor
- EDRF = Endothelium-Derived Relaxing Factors
- EDTA = Ethylene Diamine Tetraacetic Acid
- EDV = Endothelial-Dependent Vasodilation
- EF = Endothelial Function
- EI = Electron Ionization
- ELAM-1 = Endothelial Leucocyte Adhesion Molecule
- eNOS = Endothelial NO Synthase
- ESC = European Society of Cardiology
- ESH = European Society of Hypertension
- ET-1 = Endothelin-1
- ETH = Ethanol
- EAR = Estimated Average Requirement

F

- FAD = Flavin Adenine Dinucleotide
- FBF = Forearm Blood Flow
- FFQ = Food Frequency Questionnaire
- FGF-23 = Fibroblast Growth Factor-23
- FM = Fat Mass
- FMD = Flow Mediated Dilation
- FMN = Flavin Mononucleotide

G

- GCMS= Gas Chromatography Mass Spectrometry
- GP = General Practitioner

H

- Hb = Hemoglobin
- HDL = High Density Lipoprotein
- HHcy = Homocysteinaemia
- HIV = Human Immunodeficiency Virus
- HNRC = Human Nutrition Research Centre
- HPLC = High Performance Liquid Chromatography
- HR = Heart Rate
- HRP = Horseradish Peroxidase
- Ht = Hypertension

I

- ICAM-1 = Intercellular Adhesion Molecule
- ID = Identification
- Ifnγ = Interferon Gamma
- IL-6 = Interleukin 6
- iNOS = Inducible NO Synthase
- IOM = Institute Of Medicine
- IPAQ = International Physical Activity Questionnaires
- IQR = Interquartile Range
- IRAS = Integrated Research Application System
- ISH = Isolated Systolic Hypertension
- IU = International Unit

L

- LC-MS = Liquid Chromatography Mass Spectrometry
- LC-MS/MS = Liquid Chromatography–Tandem Mass Spectrometry
- LD = Laser Doppler
- LDI = Laser Doppler Iontophoresis
- LD-ION = Laser Doppler Iontophoresis
- LDL = Low Density Lipoprotein
- Lp(A) = Lipoprotein (A)
- LVH = Left Ventricular Hypertrophy
- LV = Left Ventricular

M

- MAPK = Mitogen Activated Protein Kinases
- MET = Metabolic Equivalent Medical
- Mets = Metabolic Syndrome
- MI = Myocardial Infraction
- ML = Maximum Level
- MMP = Matrix Metalloproteinases
- MRM = Multiple Reaction Monitoring
- MTH = Methanol
- MUFA = Monounsaturated Fatty Acid
- Mcg = microgram
- ME = Myalgic Encephalopyelitis

N

- NADPH = Nicotinamide-Adenine-Dinucleotide Phosphate
- NaOH = Sodium Hydroxide
- NB = Nota Bene
- NHS = National Health Service
- NIST = National Institute Of Standards And Technology
- NO = Nitric Oxide
- NO_2^- = Nitrite
- NO_3^- = Nitrate
- NOA = Nitric Oxide Analyser
- NOHA = N(Omega)-Hydroxy-L-Arginine
- nNOS = Neuronal NO Synthase
- NRES = National Research Ethics Service
- NOS = Nitric Oxide Synthase

O

- ODS = Office of Dietary Supplements
- $ONOO^-$ = Peroxynitrate
- ONT = Oral Nitrate Test
- OW = Overweight
- 25(OH)D = 25- hydroxyvitamin D
- 1, 25 (OH)D = 1,25-dihydroxyvitamin D

P

- PAD = Peripheral Arterial Disease
- PAL = Physical Activity Level
- PORH = Post Occlusive Reactive Hyperemia
- PPG = Photopletysmography
- PROSPERO = International Prospective Register of Systematic Reviews
- PTAD = 4-Phenyl-1,2,4- Triazoline-3,5-Dione
- PTH = Parathyroid Hormone
- PTT = Pulse Transit Time
- PUFA = Polyunsaturated Fatty Acid
- PWV = Pulse Wave Velocity
- PWA = Pulse Wave Analysis

R

- R&D = Research & Development
- RAAS = Renin Angiotensin-Aldosterone System
- RAS = Renin Angiotensin Aldosterone
- RBC = Red Blood Cell
- RCT = Randomized Clinical Trials
- RDA = Recommended Dietary Allowance
- REC = Research Ethics Committee
- RHI = Reactive Hyperaemia Index
- RH-PAT = Reactive Hyperaemia Peripheral Arterial Tonometry
- RL = Resting Level
- RLU = Relative Light Units
- RMP = Reference Measurement Procedure
- RNI = Reference Nutrient Intake
- ROS = Reactive Oxygen Species
- RPM = Revolutions Per Minute
- RR = Relative Risk
- RVI =Royal Victoria Infirmary
- RIA = Radioimmunoassay
- RXR = Retinoic Acid X Receptor

S

- SACN = Scientific Advisory Committee on Nutrition
- SBP = Systolic Blood Pressure
- SD = Standard Deviation
- SE = Standard Error
- SFA =Saturated Fatty Acid
- SMC = Smooth Muscle Cells
- SMD = Standardized Mean Difference
- SPSS = Statistical Package For The Social Sciences

T

- T2D = Type 2 Diabetes
- TFAA = Trifluoroacetic Anhydrate
- TH = Time to Half Decay from Maximum
- TIA = Transient Ischaemic Attack
- TM = Time to Maximum
- TMB = Tetramethylbenzidine
- TNF-A = Tumor Necrosis Factor - Alpha
- TR = Time to Recovery
- TTR = Tracer to Tracee Ratio

U

- UK = United Kingdom
- US = United State
- UVB = Ultra Violet B

V

- VCAM-1 = Vascular Cell Adhesion Molecule
- VDR = Vitamin D Receptor
- VDRE = Vitamin D Response Elements
- VDSP = Vitamin D Standardisation Program
- VEGF = Vascular Endothelial Growth Factor
- VPF = Vascular Permeability Factor
- VSM = Vascular Smooth Muscle
- VDBP = Vitamin D Binding Protein

W

- WBC = White Blood Cell
- WHO = World Health Organization

Z

$ZnSO_4$ = Zinc Sulfate

Structure of the Thesis

The first chapter of this thesis provides an introduction to the physiological role of endothelial function (EF) and vitamin D in health and diseases, and contribution to the pathogenesis of cardiovascular disease (CVD). I also discussed about the function of nitric oxide (NO) and the mechanistic link between vitamin D and NO pathway and how they influence vascular health.

In the second chapter, I conducted a systematic review and meta-analysis of randomised controlled clinical trials which examined the effects of vitamin D supplementation on physiological measures of EF (flow mediated dilatation, forearm blood flow, microvascular blood flow, pulse wave velocity and augmentation index).

In the third chapter, I discussed about various methods of measurements of NO production, and the advantage and disadvantages of each method. I also discussed about the measurement of biomarkers of EF in biological samples including the methods to measure nitrate concentrations using three methods (chemiluminescence, Griess and Gas Chromatography Mass Spectrometry (GCMS). The last part of this chapter was dedicated for the comparison of chemiluminescence vs griess methods for the measurement of nitrate/nitrite in biological samples. A validation study was also done for the analysis of serum vitamin D using dry blood spots (DBS) and conventional methods (assays on serum collected by venepuncture).

In chapter four, I reported the results of a cross sectional study on the association of vitamin D with physiological and biochemical markers of endothelial function in overweight and obese post-menopausal women.

In the fifth chapter, I report the result on the association between whole-body NO production with vitamin D. In this study, I investigated the association between age, whole-body NO production and vascular health in healthy young and old men and women. In addition, I investigated for the first time the association between whole-body NO production and vitamin D status measured by a novel, non-invasive method using stable isotope tracers.

In the final chapter, I provide a synthesis and a general discussion of the main findings, highlight the strengths and limitations of the current work and the recommendations for future research.

Chapter 1. Introduction

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is a term used to describe problems related to the functions of the heart and circulation. CVD emerged as a major public health concern in economically-developed countries in the 20th century (Dalen *et al.*, 2014), and remain associated with an enormous burden of mortality and morbidity worldwide (Frayn and Stanner, 2008). It is estimated that in 2030, there will be 23.6 million deaths due CVD (Cannon, 2013). CVD can be divided into four main types, depending on the location of the main deterioration in the vascular system which affects blood supply to the heart [coronary heart disease (CHD)], brain [stroke] and peripheral arteries [peripheral arterial disease, (PAD)] or aorta [aortic aneurysm](Cannon, 2013).

1.1.1 Coronary heart disease

CHD and coronary artery disease (CAD) are two terms that are often used interchangeably. However, CHD is a result of CAD, which is related to the accumulation of plaque in the walls of the coronary arteries that result in the thickening in the walls and limitation of the blood flow to the heart which leads to CHD **(Figure 1. 1)** (Dahlof, 2010).

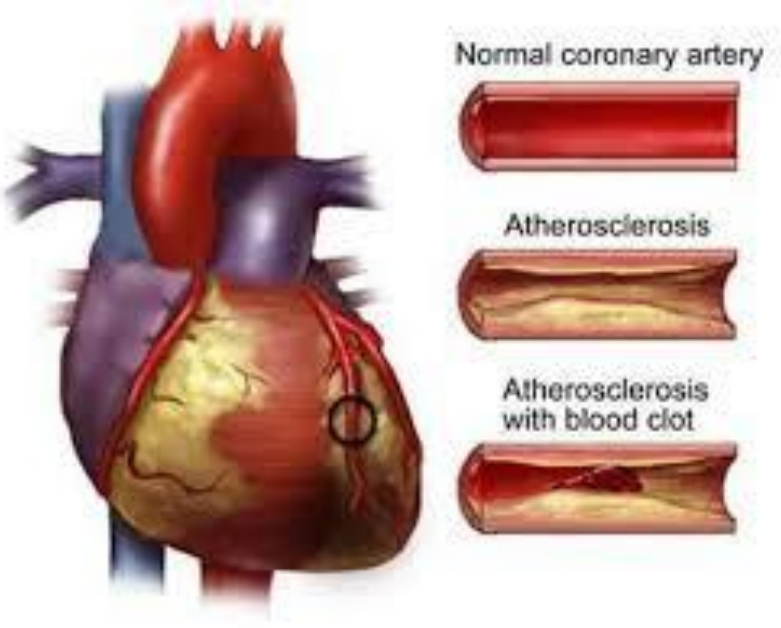


Figure 1. 1: The accumulation of plaque causes narrowing of the artery, limiting the blood flow to the heart, and leads to CHD.

Taken from (National Heart and Lung and Blood Institute, 2016a)

The process of vascular wall thickening is called atherosclerosis. Atherosclerosis is the hardening of the innermost layer of the artery, the intima, and results from the accumulation of cells, connective-tissue elements, lipids and debris which forms an atheromatous plaque (Lavie *et al.*, 2013). This plaque may obstruct blood supply to the myocardium causing symptoms of pain on exertion (angina). Occasionally, the plaque may rupture and thrombosis may lead to complete blockage of blood supply to, and death of the corresponding cardiac muscle (myocardial infarction) (Dahlof, 2010).

1.1.2 Cerebrovascular disease

Cerebrovascular disease is caused by insufficient supply of blood to the brain as the consequence of obstruction in, or rupture of, the relevant blood vessels (Elesber *et al.*, 2006). There are a number of different types of cerebrovascular disease and the four most well-known are:

1) Stroke – the condition where one part of the brain is damaged by the lack of blood supply or bleeding into the brain from a burst blood vessel.
2) Transient ischaemic attack (TIA) – the condition where blood supply to one part of brain has failed temporarily and which results in similar symptoms to stroke.
3) Subarachnoid haemorrhage – a type of stroke where blood leaks out of the brain's blood vessels on to the surface of the brain.
4) Vascular dementia – characterised as persistent impairment in mental (cognitive) ability as a consequence of stroke or other problems related with blood circulation to the brain.

1.1.3 Peripheral arterial disease

Peripheral arterial disease (PAD), also known as peripheral vascular disease, is the condition where the accumulation of fatty deposits in the arteries results in insufficient blood supply to tissues other than the heart and brain and, most frequently, affects the leg muscles **(Figure 1. 2)**. It appears as pain in the leg muscle during ambulation and the symptoms can be treated by exercise, smoking cessation and pharmacotherapy. The risk factors for PAD are the same as those for other atherosclerotic diseases and include older age and being male. Smokers have three times greater risk of PAD compared with non-smokers (Kannel and McGee, 1979; National Heart and Lung and Blood Institute, 2016b).

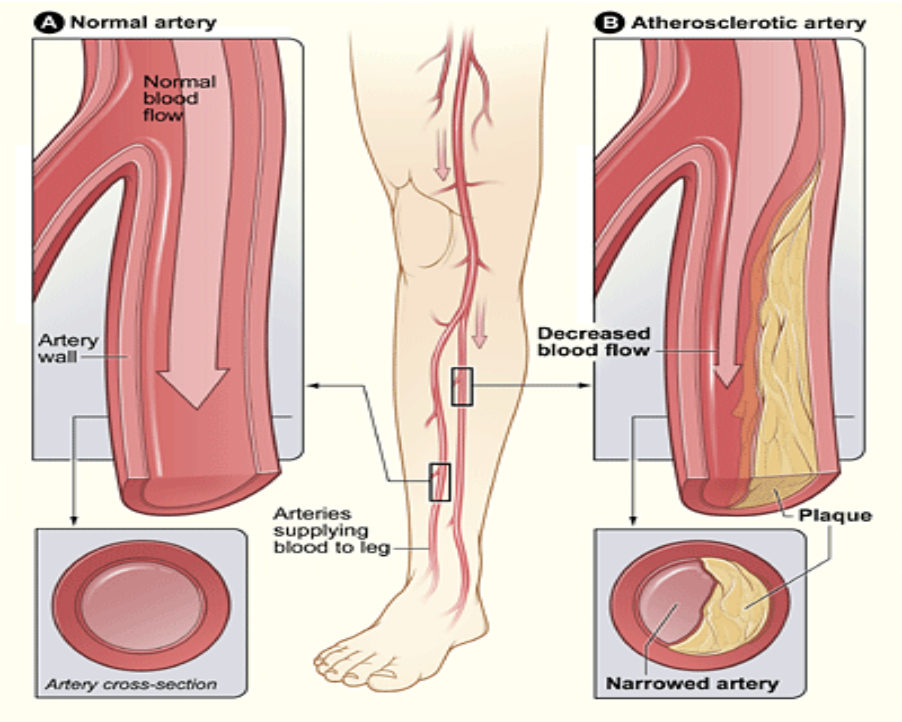


Figure 1. 2: Illustration of the occurrence of PAD.

Starting from the normal artery with normal blood flow (Figure A). The image shows a cross-section of the normal artery, which is clear from any fatty substances. Figure B shows an artery with plaque build-up that is partially blocking blood flow. The image shows a cross-section of the narrowed artery, which restricts blood supply to the leg. Taken from (National Heart and Lung and Blood Institute, 2016b).

1.1.4 Aortic aneurysm disease

Aortic aneurysm disease is the condition where enlarged area occurs in the aorta, the body's main artery. This is caused by weakness in the wall of the artery, which can lead to rupture or dissection of the aorta (National Heart and Lung and Blood Institute, 2016b). There are two most common aortic aneurysms depending on the location of the disease; thoracic aortic aneurysm (occurs in the chest) and abdominal aortic aneurysm (occurs in the abdominal area) **(Figure 1.3)**. Many risk factors contribute to the occurrence of aortic aneurysm including male gender, aging, smoking, high blood pressure and family history. However, the most common risk factor is atherosclerosis, which narrows the arteries and increases the force of blood pushing against the walls of an artery (Dahlof, 2010).

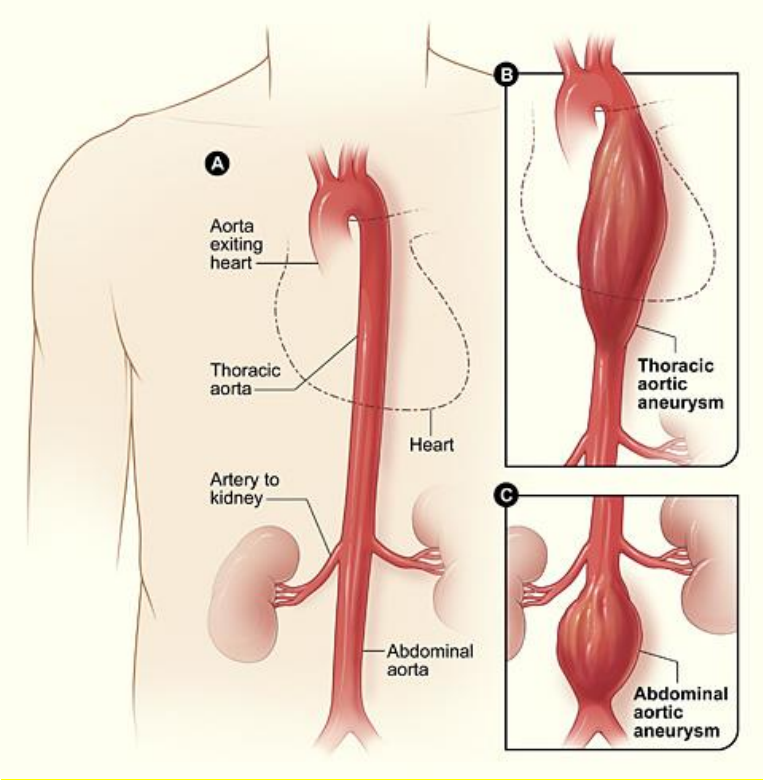


Figure 1. 3: Illustration of the occurrence of aortic aneurysm

Starting from the normal aorta exiting the heart (Figure A). The image shows a normal aorta. Figure B shows an aorta with bulging that is located behind the heart (thoracic aortic aneurysm). Figure C shows abdominal aortic aneurysm, which is located below the arteries that supply blood to the kidneys. Taken from (National Heart and Lung and Blood Institute, 2016b).

1.2 Epidemiology of cardiovascular disease

1.2.1 The burden of cardiovascular disease globally and in the UK

CVD are the leading cause of death worldwide, accounting for around 1/3 of all-cause mortality (Cannon, 2013). Approximately 7.4 million deaths are due to CHD and 6.7 million deaths to stroke (World Health Organization, 2016b).

Cardiovascular mortality varies between countries. Low CVD mortality rates are observed in Japan and Mediterranean countries such as France, Spain, Portugal and Italy, while eastern European countries such as Ukraine, Russia and Latvia experience high CVD prevalence. These differing geographical trends are probably due to differences in lifestyle including smoking patterns, nutritional transitions and changes in physical activity/ sedentary behaviour, which are important determinants of CVD risk (Institute of Medicine, 2010). According to WHO (2016), globally around 45% of CVD deaths are due to CHD **(Figure 1.4)**.

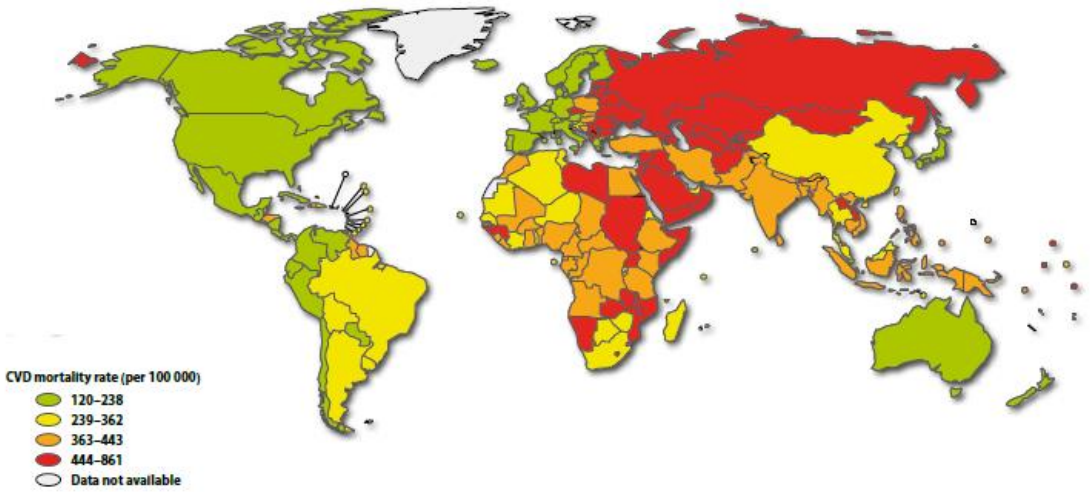


Figure 1. 4: The global distribution of CVD mortality rates (age standardized, per 100000).

Taken from (World Health Organization, 2016a).

In the UK in 2014, CVD were the second most common cause of death after cancer and accounted for 19% and 28% of premature deaths among women and men, respectively. CHD is the biggest single cause of death in the UK with one in five men and one in ten women dying from CHD. This is followed by stroke and 25% of CVD deaths are from stroke. It has been estimated that 1.5 million of the UK population have survived a heart attack and 2.6 million are currently affected by CHD (British Heart Foundation, 2015) .

1.2.2 Variation in cardiovascular disease in the UK

In the UK, CVD death rate is higher in urban and northern populations, particularly in Scotland, and lower in the south of England **(Figure 1.5)**. This geographical patterning is probably due to differences in socioeconomic status which drive differences in physical activity, smoking and dietary behaviours that are major determinants of CVD risk (British Heart Foundation, 2015).

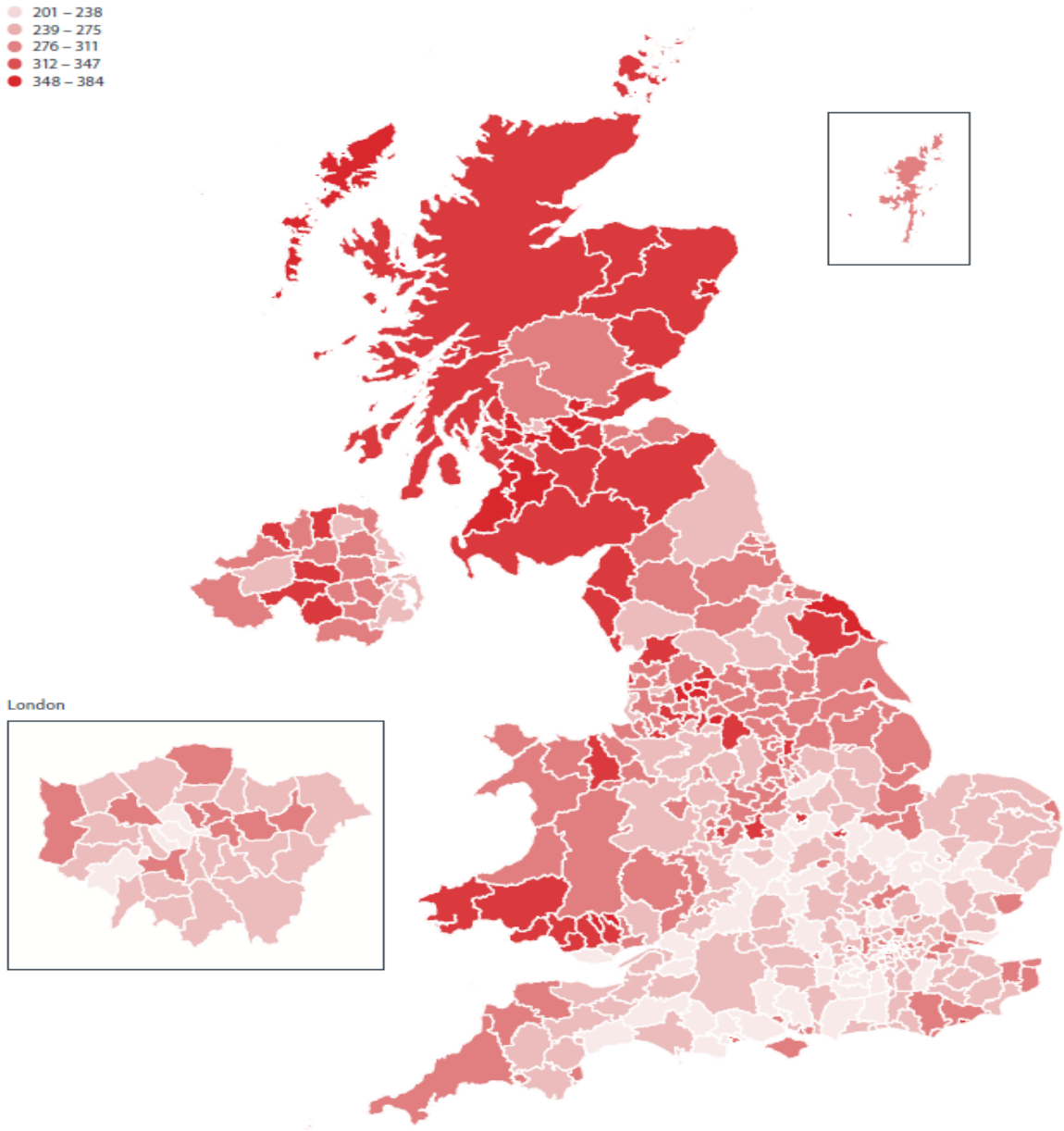


Figure 1. 5: Aged-standardized death rates from CVD in men and women of all ages, per 100 000 by local authority, United Kingdom, 2011/2013.

Taken from (British Heart Foundation, 2015)

1.2.3 Risk factors for CVD

There are many risk factors for CVD **(Table 1. 1)**, some of which are non-modifiable (such as age, sex and family history of CVD), whereas others are modifiable (such as abnormal blood lipids, smoking, diabetes, elevated blood pressure, abdominal obesity, lack of physical activity, diet, stress and overconsumption of alcohol). Modifiable factors are the major contributors to cardiovascular morbidity and mortality and account for > 90% of all myocardial infarctions (Dahlof, 2010).

Table 1. 1: Risk Factors for CVD-(World Health Organization, 2016a)

Modifiable (lifestyle)	Potentially modifiable (Biochemical and physiological)	Non-modifiable
• Cigarette smoking • Diet rich in saturated fats and energy with low intake of fruits and vegetables. • Physical inactivity • Excess alcohol intake • Psychosocial stress • Obesity	• Dyslipidaemias (high plasma cholesterol, low plasma HDL, high triglycerides) • High blood pressure • Diabetes mellitus • Thrombotic factors • LVH/LV mass • Lp(A) • Haematological - Reductions in hematocrit and polymorphonuclear leukocytes (Jenkins *et al.*, 2007) • Oxidized lipids	• Age • Gender • Genetic factors

Lp(A), lipoprotein (A); LVH, Left ventricular hypertrophy; HDL, high density lipoprotein

1.3 Atherosclerosis

1.3.1 Definitions

Atherosclerosis is a multifactorial, progressive lesion of the arterial wall due to the accumulation of connective tissue components, cholesterol, macrophages, proliferation of smooth muscle cells (SMC) and the formation of thrombus (Whayne, 2011). The clinical outcomes of coronary heart, cerebrovascular, and peripheral vascular diseases are largely attributed to atherosclerotic lesions. Endothelial cells, leukocytes and intimal smooth muscle cells are the key roles of the development of atherosclerosis. Plaque causes affected arteries

to harden and narrow which is potentially dangerous due to restricted blood flow which can damage organs and cause malfunction. The atherosclerotic process may commence silently in late adolescence with the clinical manifestations usually becoming obvious after the age of 45 years (Wilson, 2004).

1.3.1.1 Pathogenesis and Stages of Atherosclerosis

One of the earliest stages of the atherosclerosis process is deterioration of endothelial function (EF) **(Figure 1.6)**. It has been suggested that reduced nitric oxide (NO) bioavailability is one of the factor responsible for this deterioration (Bondonno *et al*., 2016). The process may begin in childhood or early adulthood. The accumulation of lipoproteins in foam cells that leads to the formation of yellow- white fatty streaks in the major arteries is the first step into atherosclerosis. Fatty streaks are composed mainly of smooth muscle cells, saturated with cholesterol and monocyte-derived macrophage-like foam cells (Libby *et al.*, 2002; Weber and Noels, 2011). The development of fatty streak starts when the accumulation of oxidised LDL in the vascular wall activates inflammatory reactions, which in turn, leads to secretion of cytokines. These cytokines then attract T cells and leukocytes to the site of injury, and lead to the accumulation of fat in macrophages which form lipid-laden foam cells and serve as the hallmark of early stage atherosclerotic lesion formation (Rosenfeld, 2013). Although fatty streaks *per se* do not cause any major problems related to heart function they can develop into atherosclerosis in the long term.

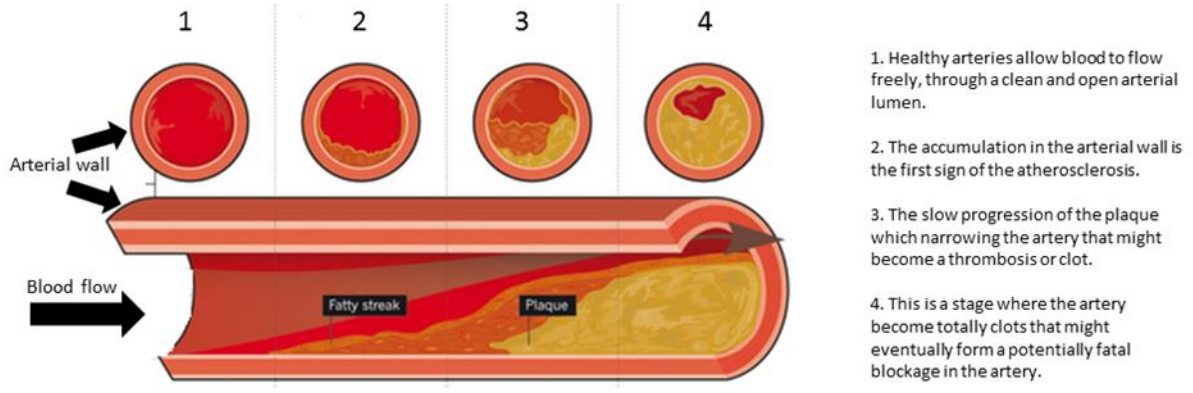


Figure 1. 6: The process of atherosclerosis

Modified from (Libby *et al.*, 2011)

The formation of fibrous plaque in the walls of the arteries is the second stage of atherosclerosis. Plaque is composed of a large number of macrophages, smooth muscle cells and lymphocytes (Libby *et al.*, 2002) and narrows the artery lumen and hardens the artery wall. Atherosclerotic plaques can be classified into two forms, depending on how they progress in the arteries. The slowly progressing plaques tend to be more stable and are not prone to rupture. On the other hand, more rapidly growing plaques have a thin fibrin cap and are more susceptible to rupture, which increases the risk of thrombotic events **(Figure 1.7)**.

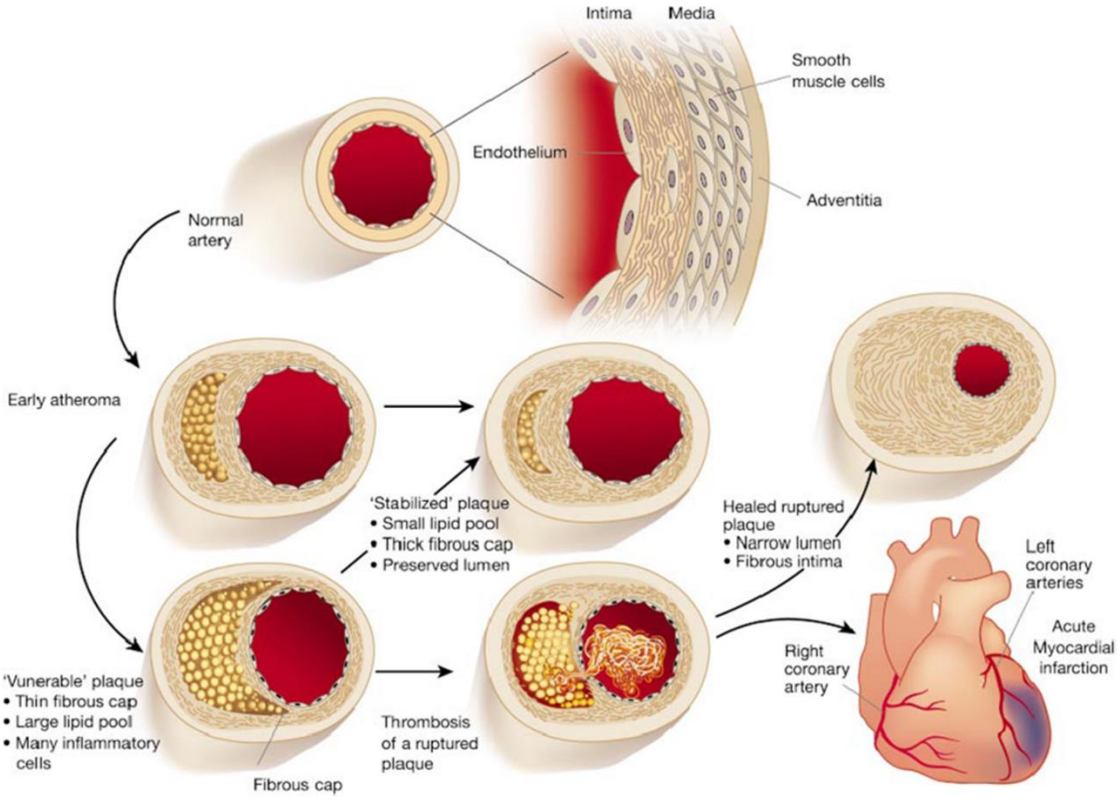


Figure 1. 7: Stable and unstable plaque

Taken from (Libby *et al.*, 2011)

The final stage of atherosclerosis begins when a fibrous plaque ruptures, revealing the cholesterol exposed to injury or stress, thereby provoking intense reactions of blood clotting and resulting the formation of thrombus. Depending on the location of thrombus, this condition will lead to heart attack or stroke if the thrombus is large enough to block the coronary blood vessel or cerebral blood vessel, respectively (Rosenfeld, 2013). Approximately 76% of all fatal cases related to coronary thrombi are caused by the plaque ruptured (Falk et al, 2006).

1.3.2 Endothelial function in health and disease

1.3.2.1 Functions of the endothelium

The endothelium is the single inner layer of squamous cells lining the blood vessel which separate the vascular lumen from the rest of the blood vessel. It is believed to be the key vessel wall component in the initiation of the atherogenic process. The endothelium can be considered as one of the major organs of the human body with surface area extending about 350m^2 and weighing approximately of 110g (Pries *et al.*, 2000).

Endothelial cells line the entire vascular system, from the heart to the smallest capillaries (Alberts *et al.*, 2002). The structure of the endothelial cell is important in the maintenance and integrity of the circulatory function and vessel wall. The semi-permeable characteristic of these cells controls the passage of materials into and out of the blood vessels. Endothelial cells secrete several vasoactive agents including the vasodilators nitric oxide (NO), prostacyclin and Endothelium-Derived Hyperpolarizing Factor (EDHF) and the vasoconstrictors endothelin-1 (ET-1), angiotensin II (AngII), and thromboxane A_2. Perturbation of endothelial structure or function will lead to haemodynamic instability, which can result in endothelial injury and dysfunction. These events may initiate many pathological process such as atherosclerosis, increase the risk of thrombosis and disturb permeability of the blood vessel (Sumpio *et al.*, 2002).

1.3.2.2 Factors affecting endothelial function

1. Nitric oxide (NO)

   NO is a water soluble gas which is produced continuously in endothelial cells from its precursor L-arginine via the enzymatic action of endothelial NO synthase (eNOS). It is a highly diffusible molecule with a very short half-life (~6-30 sec) (Palmer *et al.*, 1988). An alternative source of NO is the non-enzymatic reduction of dietary inorganic nitrate to nitrite by oral bacteria; nitrite can then be further reduced to NO in the gastric lumen (in the presence of low oxygen tension and low pH) and in the blood vessels (reaction with deoxygenated haemoglobin or other enzymes with reductase activity e.g. xanthine oxido-reductase or aldehyde dehydrogenese). NO is considered to be the key mediator produced by the endothelial cell for the control of vascular tone and plays a pivotal role in maintaining vascular homeostasis. The functions of NO in endothelial cells include: relax blood vessels, inhibit platelet aggregation and adhesion, limit oxidation of low

density lipoprotein (LDL) cholesterol, inhibit proliferation of vascular smooth muscle cells and decrease inflammatory responses (Forstermann, 2010). Decreased production of NO and/or increased NO inactivation leads to endothelial dysfunction (Higashi *et al.*, 2009), and this precedes the development of atherosclerotic disease.

2. Oxidative stress

Oxidative stress contributes to the development of cardio-metabolic diseases including atherosclerosis, hypertension, dyslipidemia, diabetes, myocardial infarction, heart failure and angina pectoris (Schächinger *et al.*, 2000). Atherosclerosis may occur as a result of reduced endothelium-derived NO and from increased production and/or impaired inactivation of reactive oxygen species (ROS) (Lavie *et al.*, 2013). ROS are essential for normal cell function (Sena and Chandel, 2012), but adequate levels of antioxidant defences are required to avoid the harmful effects of excessive ROS production. An imbalance of NO and ROS, so-called oxidative stress, contributes to endothelial dysfunction through the inactivation of NO (Hsieh *et al.*, 2014). Elevated levels of ROS reduce the availability of NO by reacting with NO to form peroxynitrite, which leads to many pro-atherogenic events (Schulz *et al.*, 2011; Hsieh *et al.*, 2014).

3. Ageing

Advancing age is a major risk factor for CVD due to age-related impairment of EF (North and Sinclair, 2012). Decreased availability of NO occurs during ageing due to greater ROS production (e.g. superoxide) without a compensatory increase in antioxidant defenses and this lower NO contributes to reduced peripheral artery endothelium dependent dilation (EDD) (Palmer *et al.*, 1988).

4. Health status

The ability of the endothelium to maintain the integrity of the vessel wall can be affected by the biochemical and pathophysiological state of the body due to both exogenous and endogenous factors including:

Chronic smoking: Active and passive smoking may deteriorate EF due to decreased formation of NO, increased NO degradation via generation of oxygen free radicals, increased formation of asymmetric dimethylarginine (ADMA) and greater ROS (Toda and Toda, 2010).

Obesity: The reduced NO encountered in obesity arises either because of enhanced degradation (oxidative stress) or impairment of endothelial NO synthase activity (eNOS inactivation). This leads to impaired EF due to increased vasoconstrictor factors, such as endothelin-1, and sympathetic nerve activation (Toda and Okamura, 2013).

Hypercholesterolemia: In hypercholesterolemia, impaired NO activity has been associated with increased NO degradation by oxygen radicals (Kuwai and Hayashi, 2006). Hypercholesterolemia increases production of the superoxide anion, which may be responsible for an impairment of the balance between vasorelaxant and vasoconstrictor factors in the endothelium (Katusic, 1996; Perrault *et al.*, 2000). Elevated ADMA is observed in people with hypercholesterolaemia (Böger *et al.*, 1998; Landim *et al.*, 2009; Sydow and Böger, 2012). ADMA is well known as an endogenous competitive inhibitor of NO synthase, and thus may cause endothelial dysfunction by reducing availability of NO (Sibal *et al.*, 2010).

Hyperglycaemia: Transient and prolonged hyperglycaemia impair EF. Hyperglycaemia is associated with increased oxidative stress, uncoupling of eNOS, LDL oxidation, and increased concentrations of vasoconstrictor mediators e.g. endothelin, angiotensin, and prostanoids (Sena *et al.*, 2013) - all of which impair endothelium-dependent vasodilation.

High blood pressure: Hypertensive patients have impaired EF (Puddu *et al.*, 2000). This dysfunction may be due to reduce NO availability (increased degradation by oxidative stress and reduced production by eNOS inactivation) and abundance of vasoconstrictor agents in the circulation, such as angiotensin II and prostaglandins (Tang and Vanhoutte, 2010), which causes an imbalance between vasodilation and vasoconstriction.

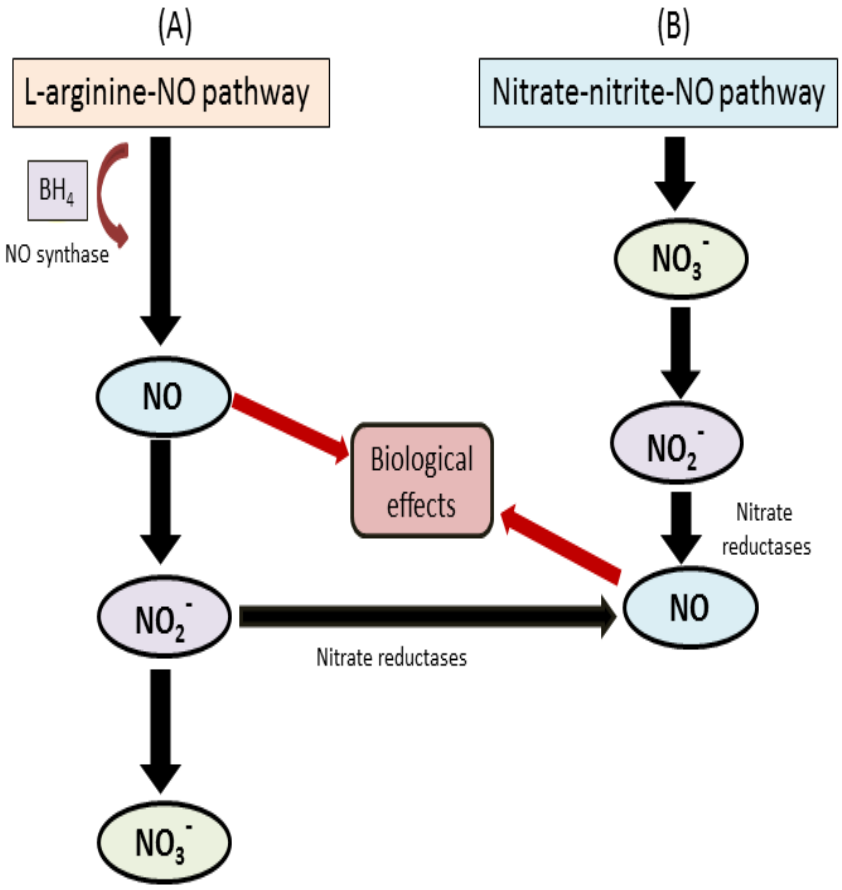
Homocysteinaemia: Homocysteinaemia (HHcy) is a pathological condition characterized by raised plasma concentration of total homocysteine (Clarke *et al.*, 1991). Homocysteine is a thiol amino acid synthesized during the metabolic conversion of methionine to cysteine by the demethylation pathway (Lai and Kan, 2015). Possible mechanisms by which raised homocysteine concentration causes

endothelial dysfunction include NO inhibition, endothelin-1 (ET-1) induction, EDHF inhibition, proliferation of smooth muscle cells, increased oxidative stress and promoting inflammation (Pushpakumar *et al.*, 2014).

Hormones: Hormones that increase NO production include estrogen, progesterone, insulin, and growth hormone. In contrast, other hormones, including glucocorticoids, progesterone, and prolactin, decrease NO availability (Duckles and Miller, 2010). Long-term exposure to aldosterone reduced the production of tetrahydrobiopterin (BH_4) and increased oxidative stress (Hashikabe *et al.*, 2006). Elevated aldosterone concentrations lead to the development of hypertension due to oxidative stress (Queisser and Schupp, 2012). Oestrogen modulates NO release by the endothelial cell (Vanhoutte *et al.*, 2009) by up regulating eNOS activity via a receptor-mediator system (Hayashi *et al.*, 1995).

1.4 The Production of Nitric Oxide in humans

NO is considered to be the key mediator produced by the endothelial cell for the control of vascular tone by activating guanylyl cyclase and, therefore, is one of the most important molecules secreted by the endothelium (Forstermann, 2010). There are two NO synthase-dependent pathway in humans; either it can arise as an end product of the L-arginine-NO pathway or be produced from dietary nitrate as summarised in **Figure 1.8**.



NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; BH_4, tetrahydrobiopterin

Figure 1. 8: NO synthase-dependent pathway in human

NO synthase-dependent pathway in human from (A) L-arginine-NO pathway or (B) Enterosalivary nitrate-nitrite-NO pathway - figure adapted from (Gilchrist *et al.*, 2011)

1.4.1 L-arginine - NO pathway

NO can be generated endogenously by three isoforms of the enzyme NO synthase: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Forstermann and Sessa, 2012). eNOS is a homodimeric enzyme which is expressed constitutively in endothelial cells. It oxidizes and converts the amino acid L-arginine into L-citrulline and NO (Michiels, 2003). There are two steps required in the production of NO. The first step is the hydroxylation of L-arginine to N^{ω}-hydroxy-L-arginine by NOS. In the second step, NOS oxidizes N^{ω}-hydroxy-L-arginine to L-citrulline and NO (Stuehr *et al.*, 2001; Bruckdorfer, 2005; Forstermann and Sessa, 2012). This process requires molecular oxygen and nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates. Many cofactors are needed in this process including calmodulin-calcium, tetrahydrobiopterin (BH_4), haem, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Forstermann and Sessa, 2012).

1.4.2. Entero-salivary nitrate-nitrite-NO pathway

NO can also be produced by the entero-salivary nitrate-nitrite-NO pathway which includes the conversion of nitrate (NO_3^-) to nitrite (NO_2^-) to NO (Bondonno *et al.*, 2016). Rich dietary sources of NO_3^- include vegetables such as cress, celery, lettuce, red beetroot, chervil, spinach or rocket (Hord *et al.*, 2009). Nitrate from the diet or endogenous sources is reduced to NO_2^- by the commensal bacteria in the oral cavity and gastrointestinal tract **(Figure 1.9)**. After the formation of NO_2^-, there are innumerable pathways in the body for its further reduction to NO because of the acidity environment in the stomach, involving enzymatic and non-enzymatic pathways such as haemoglobin, myoglobin, xanthine oxidoreductase, ascorbate, polyphenols and protons (Lundberg *et al.*, 2008). It is well recognized that nitrate and nitrite act as storage pools for NO, and can be recycled back to NO, especially under hypoxic conditions where the NOS enzymes may be dysfunctional (Lundberg et al., 2008).

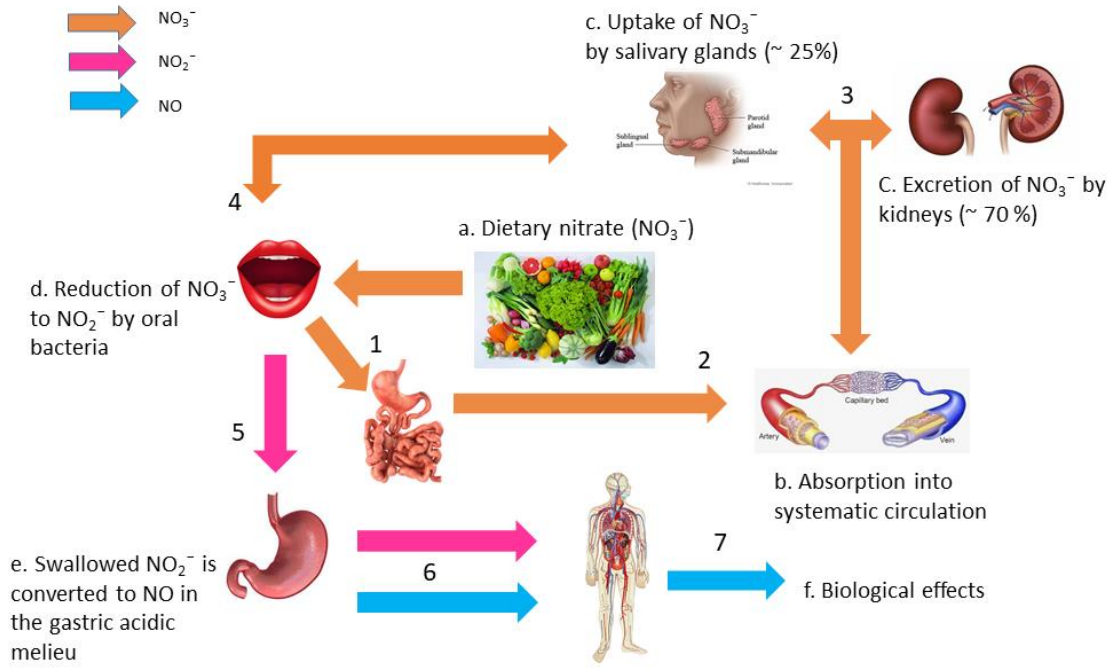


Figure 1. 9: The entero-salivary pathway in humans.

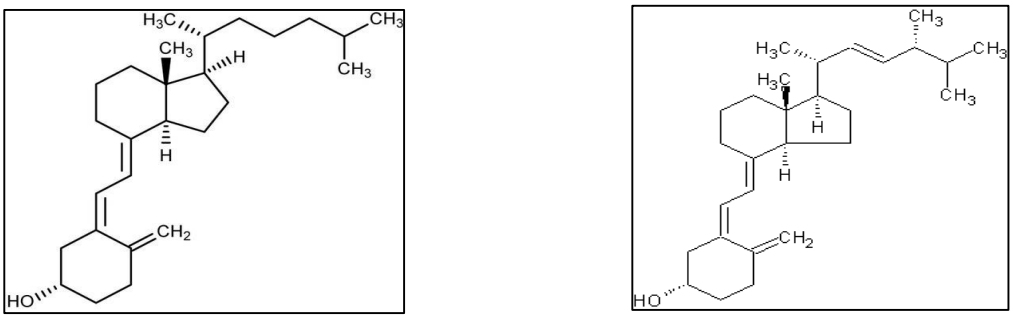
The entero-salivary pathway in human. Ingested NO_3^- (a) from dietary sources such as green leafy vegetables are swallowed and rapidly absorbed in the gastrointestinal tract (1) into the systemic circulation (2), where it mixes with endogenous nitrate. Most NO_3^- is excreted by the kidney (70%), (3) while a small portion is taken up by the salivary glands (25%) (c). Nitrate-rich saliva is secreted into the oral cavity (4) where facultative anaerobic bacteria *(E. coli, Salmonella enterica serovar Typhimurium* and *Paracoccus pantotrophus)* reduce nitrate to NO_2^- (d) via the action of nitrate reductase enzymes. Nitrite-rich saliva is then swallowed (5) and further metabolized to nitric oxide (NO) non-enzymatically because of the acidic condition in the stomach (e). NO exerts various biological effects in the body (6-7). Figure adapted from (Monaco, 2016).

1.5 Vitamin D

1.5.1 Chemical structure, preforms and metabolites of vitamin D

Vitamin D is classified as one of the fat soluble vitamins, together with vitamin A, E and K. It is also known as the sunshine vitamin that can be synthesized in the skin epidermis as a prohormone from the cholesterol-like precursor, 7-dehydrocholesterol (7-DHC) in response to ultraviolet light (290 -315 nm) (Bouillon *et al.*, 2008). Also classified as a secosteroid, vitamin D comes in two forms: vitamin D_2 (ergocalciferol) that is derived from plants and fungi such as mushrooms and vitamin D_3 (cholecalciferol) that is derived from animal sources – the latter is considered the most important source of vitamin D in most human diets (Holick, 2007) (Kassi *et al.*, 2013).

Vitamin D_2 differs structurally from vitamin D_3 in the side chain attached to the secosteroid skeleton. Vitamin D_2 contains an additional methyl group on carbon atom 24 and a double bond between carbon atoms 22 and 23 (Norman, 2008) **(Figure 1.10)**. Supplemental vitamin D_3 was more effective at raising serum 25(OH)D levels than supplemental vitamin D_2 (Tripkovic, 2013). This may be because the conversion of vitamin D_3 to 25(OH)D_3 is five times faster than that of vitamin D_2 to form 25(OH)D_2 (Houghton and Vieth, 2006; Tripkovic *et al.*, 2012).



a) Vitamin D_3 (cholecalciferol) b) Vitamin D_2 (ergocalciferol)

Figure 1. 10: Chemical structure of vitamin D

1.5.2 Metabolism of vitamin D

At higher latitudes, vitamin D stores are produced during summer, and in the winter, vitamin D status depends on vitamin D storage in the body (largely in liver and adipose tissue) and on vitamin D supplied by foods (Vaidya, 2013). In the UK, the action of UVB from the sunlight on skin is the main source of 25(OH)D concentration during spring and summer (late March/ early April to September). However, in autumn and winter season (October and early March), when the sunshine is limited, there is no ambient ultraviolet sunlight of the appropriate wavelength for skin synthesis of 25(OH)D concentration (NICE, 2016). Storage of sufficient 25(OH)D concentration in the adipose tissues and liver is very important during the winter to prevent deficiency of 25(OH)D concentration if the dietary intake of this vitamin is insufficient (O'Connor, 2011).

The skin synthesis of 25(OH)D concentration begins when the sunlight containing UVB radiation strikes the epidermis of the skin, which contains 7-DHC, to produce previtamin D_3. Prolonged UVB exposure results in conversion of previtamin D_3 to lumisterol and tachysterol which are biologically inactive (Holick *et al.*, 1980). Previtamin D_3 then undergoes spontaneous thermal rearrangement to cholecalciferol i.e. vitamin D_3. 25(OH)D concentration (from skin synthesis or after absorption from the gut) is transported to the liver bound to vitamin D binding protein (DBP) (Bhan, 2014), where the enzyme 25-hydroxylase (cytochrome P450 2R1 [CYP2R1]) catalyses hydroxylation at position 25 and the production of 25-hydroxyvitamin D [25(OH)D] or calcidiol, the most commonly used index of vitamin D status and the major circulating form of vitamin D (Haddad, 1995). From the liver, 25(OH)D is transported to kidney, again bound to DBP. In the kidney, the enzyme 1-α-hydroxylase (CYP27B1) converts serum 25(OH)D to serum 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] or calcitriol, which is the active metabolite of vitamin D **(Figure 1.11)** (Christakos *et al.*, 2012). The biological half-life of serum $25(OH)D_3$ is 20-30 days and for $1,25(OH)_2D$ is 4-6 hours (Haddad *et al.*, 1993), and this explains why serum 25(OH)D is a better indicator for assessing vitamin D status than is $1,25(OH)_2D$ (Holick, 1996).

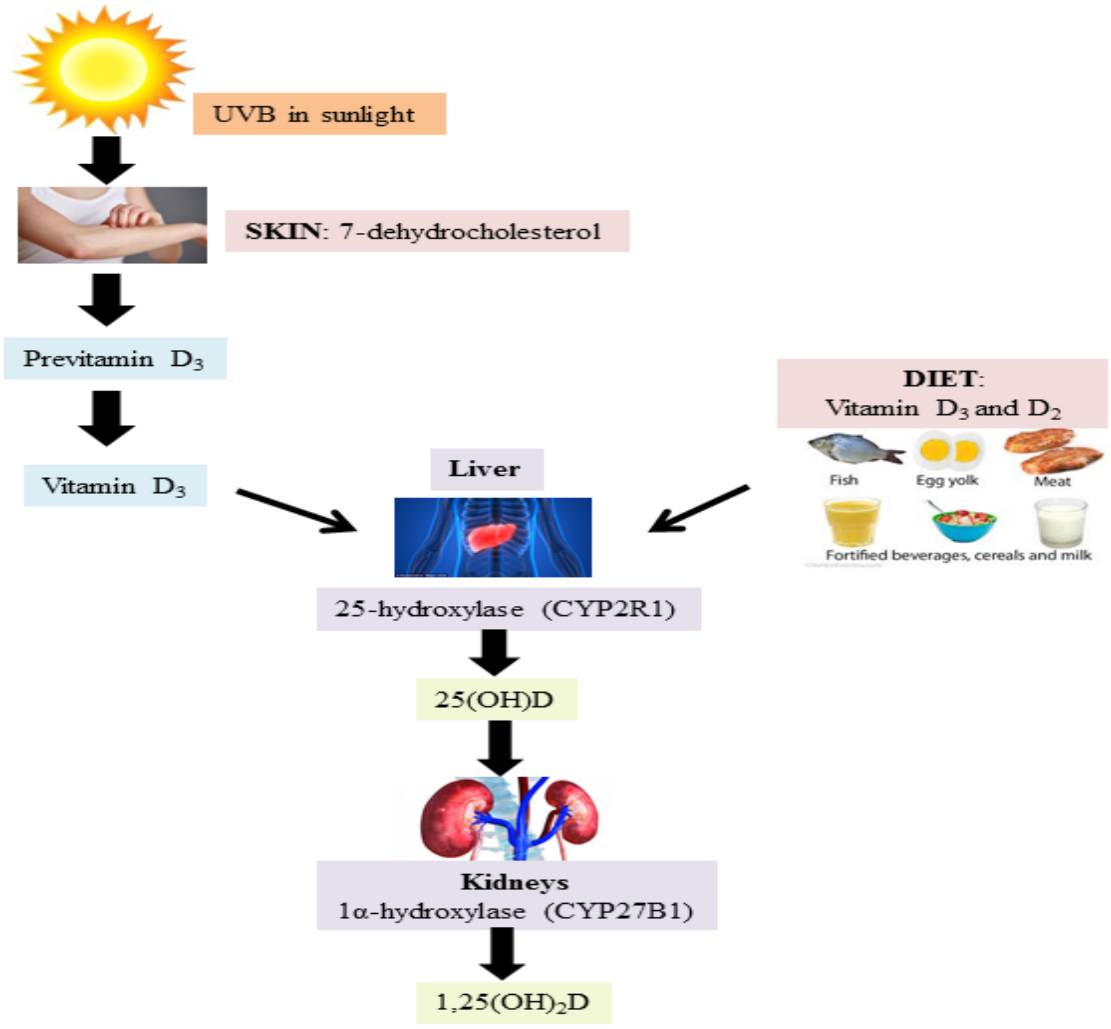


Figure 1. 11: Overview of metabolism of vitamin D.

Figure adapted from (SACN, 2016)

1.5.3 Absorption and transport of vitamin D

The absorption of vitamin D from the gut requires three organs; the liver (which synthesises bile acids), the pancreas (which produces lipases and colipase) and the small intestine (Christakos *et al.*, 2011). Ingested vitamin D is absorbed from the jejunum and ileum and transported together with lipoproteins in chylomicron *via* the lymphatic system. Therefore, partial gastrectomy, chronic pancreatic insufficiency, intrinsic small bowel disease, disorders of the biliary tract, and surgical bypass procedures of the jejunum and ileum can all affect the absorption of vitamin D (Bikle, 2007).

1.5.4 Functions and mechanisms of action of vitamin D

In addition to its classical actions in the regulation of serum calcium and phosphorus concentrations (mineral homeostasis), 1,25 dihydroxyvitamin D (1,25 (OH)D) also participates in non-classical actions, i.e. control of metabolism, cellular growth and immune function (non-skeletal functions). These effects of 1,25 (OH) D are exerted by both genomic and non-genomic mechanisms. Genomic mechanisms are responsible for long-term modulation of gene expression which is mediated through vitamin D receptor (VDR), dependent transcriptional effects in the cell nucleus (Jurutka *et al.,* 2001). Non genomic mechanisms include short term activation of intracellular signalling pathways, when the VDR induces rapid signalling, situated on the cell membrane and/or cytoplasm (Trochoutsou *et al.*, 2015) .

The genomic actions of vitamin D are mediated by the VDR which, on binding the hormone, interacts with the DNA to induce or inhibit gene expression and, ultimately, new protein synthesis (Figure 1.15). When vitamin D gets into the cell nucleus, it binds to VDR and induces a conformational modification that allows it to form a complex with the retinoic acid X receptor (RXR). Then, the VDR-RXR heterodimers bind with specific enhancer elements in DNA, known as vitamin D response elements (VDRE), a pre-defined promoter DNA sequence. The type of the co-regulatory protein (whether co-activators or co-repressor) that bind to the VDR-RXR-VDRE determine whether gene transcription is repressed or initiated (Haussler *et al.*, 2011). Because of the complexity of the signalling and transcription process related to the VDR signalling, genomic functions of vitamin D can take several hours to produce their effects (Trochoutsou *et al.*, 2015).

The non-genomic actions of vitamin D are mediated by the VDR located within caveolae (small invaginations) in the plasma membranes (mVDR), in specific locations involved in the signal transduction pathways (Pusceddu *et al.*, 2015) **(Figure 1.12)**. These are 'rapid response pathways' which are mediated within seconds to minutes compared with genomic actions which may take hours. The signalling induced by non-genomic action includes the opening of ion channels, the induction of second messenger molecules (e.g. mitogen activated protein kinases (MAPK)), and secretion of certain hormones such as estrogen, progesterone, testosterone, corticosteroids, and thyroid hormone (Haussler *et al.*, 1995; Bikle, 2014). The genomic and non-genomic pathways operates in several cell types, suggesting that 'cross talk' might happen between these two pathways (Haussler *et al.*, 2011; Dalan *et al.*, 2014).

VDR is an intracellular receptor protein, which ligand-activated transcription factor binding to vitamin D response elements in the promoter region of target genes (Andrukhova *et al.*, 2014). The VDR gene is expressed in more than 30 tissues including smooth muscle cells, pancreatic β-cells, monocytes, keratinocytes, suggesting that VDR may be involved in modulating numerous metabolic processes beyond regulation of mineral homeostasis (Nagpal *et al.*, 2005), supporting a role of vitamin D in delineating healthy trajectories of ageing.

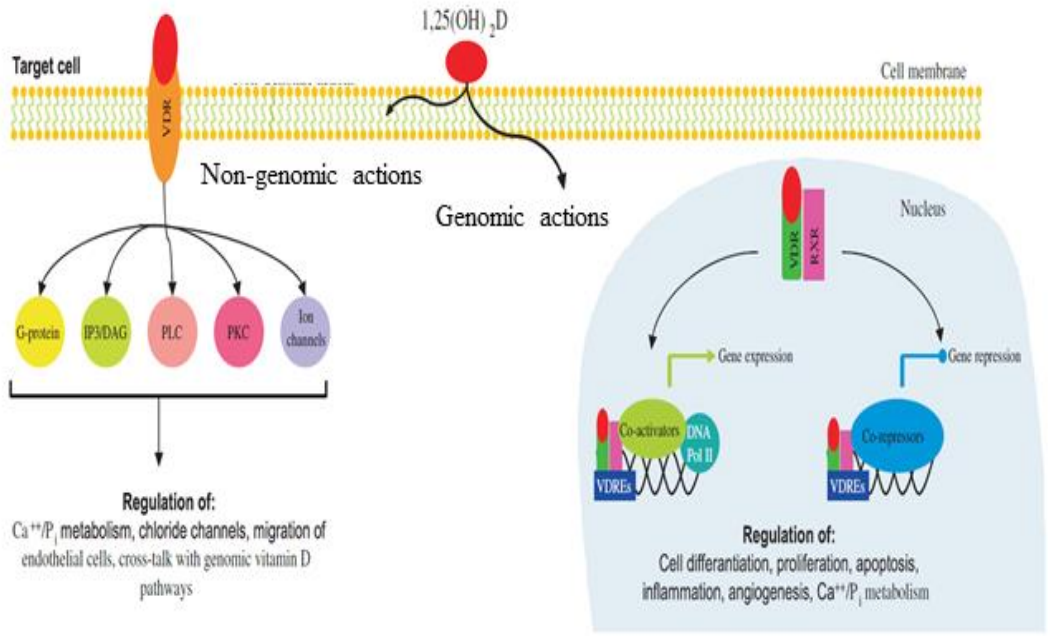


Figure 1. 12: 1,25(OH)$_2$D exerts its biological functions via non-genomic and genomic actions.

Modified from (Pusceddu et al., 2015)
DNA Pol II, DNA polymerase II; IP3/DAG, inositol triphosphate/diacylglycerol; PKC, protein kinase C; PLC, phospholipase C; RXR, retinoid X receptors; VDR, vitamin D receptor; VDREs, vitamin D response elements.

1.5.5 Daily requirement and dietary sources of vitamin D

Vitamin D is absorbed from the gastrointestinal tract from dietary sources such as fatty fish, red meat, fortified margarines and egg yolk. In addition, other meats, chicken, inner organs, dairy products and some wild mushrooms contain small amounts of vitamin D (Campos-Outcalt, 2013). The absorption of vitamin D from supplements in humans varies between 60-90%, but it has been suggested that the absorption of vitamin D from natural sources is less efficient (van den Berg, 1997).

Defining adequacy of 25(OH)D concentration status is challenging as there is no consensus among researchers as to which cut-off levels of serum 25(OH)D should be applied (Palacios and Gonzalez, 2014). The discrepant opinion of the terms used for classification such as 'suboptimal', hypovitaminosis', 'deficiency', 'insufficiency' on the 25(OH)D concentration status has bring uncertainties and confusion surrounding the dietary reference intake (DRI), estimated average requirement (EAR) and recommended nutrient intake (RNI) values as well as to identify threshold effects and possible adverse effects of excessive consumption. The Institute of Medicine (IOM) used bone health as a parameter for developing the Recommended Dietary Allowances (RDAs) of 25(OH)D concentration, and proposed that for optimum health, serum 25(OH)D should be at least 50 nmol/L (*Dietary Reference Intakes for Calcium and Vitamin D*, 2011; Ross *et al.*, 2011) . Individuals with the serum 25(OH)D <30 nmol/L are classified as deficiency, and those with 25(OH)D concentrations of 50 nmol/L and above were classified as sufficient. The Endocrine Society Task Force on serum 25 (OH)D concluded that 50 nmol/L of serum 25(OH)D is the cut-off level for 25(OH)D concentration sufficiency and in addition for the maximum benefits of 25(OH)D concentration on calcium, bone and muscle metabolism, serum 25(OH)D concentrations should exceed 75 nmol/L (Holick *et al.*, 2011). Concentrations of 25(OH)D below 25 nmol/L are considered deficient while concentrations less than 50 nmol/L are considered insufficient by the UK's Scientific Advisory Committee on Nutrition (SACN, 2016).

In the UK, the Reference Nutrient Intake (RNI) was developed for specific group at risk of insufficient sunshine exposure: infants and children aged 0-3y (UK Department of Health, 1998). However, in their more recent report, SACN have set the RNI of 10 micrograms vitamin D per day, throughout the year for everyone in the general population aged 4 years and older (SACN, 2016). Recommended intakes for vitamin D are presented in **Table 1. 2**. Intakes in this section are compared with the RDA IOM for other population groups.

Table 1.2: Vitamin D requirements by age category

Age category	IOM RDA[1]	UK RNI[2]
0 – 6 months	*	340 IU (8.5 mcg)
7 months – 3 years	600 IU (15 mcg)	280 IU (7 mcg)
14-18 years	600 IU (15 mcg)	400 IU (10 mcg)
19-50 years	600 IU (15 mcg)	400 IU (10 mcg)
51 – 70 years	600 IU (15 mcg)	400 IU (10 mcg)
>70 years	800 IU (20 mcg)	400 IU (10 mcg)
Pregnant and breast-feeding women	600 IU (15 mcg)	400 IU (10 mcg)

RDA= Recommended Dietary Allowance, RNI = Reference Nutrient Intake. IOM= Institute of Medicine,

[1](*Dietary Reference Intakes for Calcium and Vitamin D*, 2011); [2](SACN, 2016)

1.5.6 Global vitamin D status

High prevalence of vitamin D deficiency even those residing near the equator, has been a major public health problem **(Figure 1.13)** (Palacios and Gonzalez, 2014). Despite of having adequate sunshine all around the year, high prevalence of 25(OH)D concentration insufficiency (<50 nmol/L) was found in the Asian continent and majority of countries were found to have less than the optimum level of 25(OH)D concentration (75 nmol/L) (Wahl *et al.*, 2012). However, due to the unavailability of relevant data, the prevalence of vitamin D deficiency is not known in certain region such as Central America, South America (with the exception of Brazil) and much of Africa. This information gap needs attention in view of the importance of vitamin D for health.

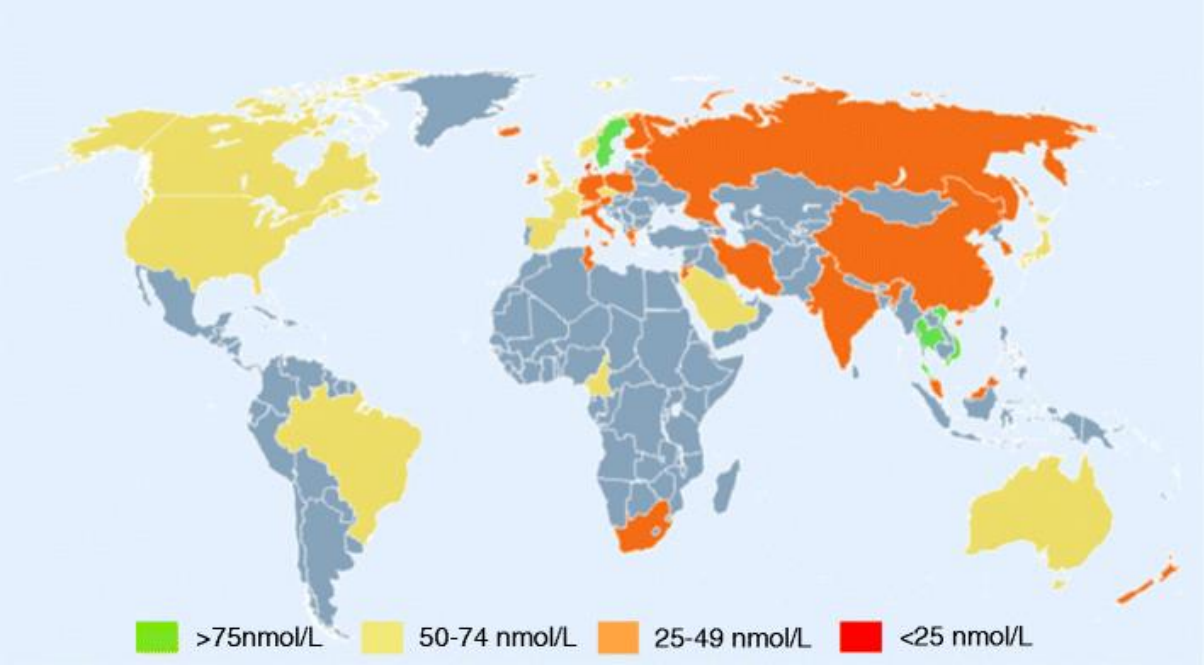


Figure 1. 13: Map of global Vitamin D status (serum 25(OH)D [nmol/L]) in adults (18+yrs).

Mean 25(OH)D values calculated from winter measurements. Taken from (Wahl *et al.*, 2012)

1.5.7 Vitamin D status in the UK

There have been concerns in recent years that vitamin D deficiency is becoming more common in Western countries including the UK. A typical normal range of serum 25(OH)D in adults living in UK is 47.4 ± 23.8 nmol/L (National Diet and Nutrition Survey, 2011). Located at latitudes >50° N, most of the UK land mass receives insufficient UVB rays from sunlight especially during October to March to allow adequate dermal synthesis of 25(OH)D concentration (Webb, 2009) thus increasing the reliance on dietary sources of the vitamin. Dietary intakes of vitamin D are low in the UK particularly in children aged 1.5 to years 3 years, and adults over 65 years old. Moreover, most adults failed to meet the UK RNI of 10 µg/day (SACN, 2016).

A cross sectional study done by (Hypponen and Power, 2007) revealed that prevalence of hypovitaminosis D in the general population in the UK was alarmingly high especially in winter and spring, where limited sunshine occurs which prevents synthesis of 25(OH)D concentration **(Figure 1.14)**. Even during the remainder of the year, cloud cover can block up to 99% of 25(OH)D concentration production. Furthermore, dietary intake of vitamin D may

be low in Great Britain. Obese participants and those living in Scotland were at the highest risk of hypovitaminosis D.

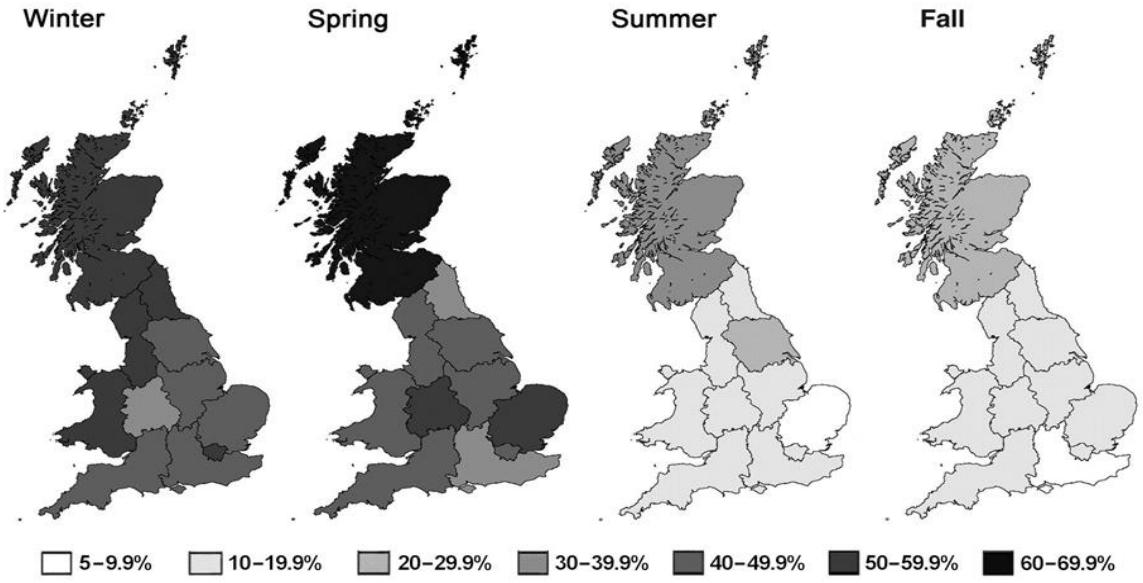


Figure 1. 14: Seasonal and geographical variation in the prevalence of hypovitaminosis D (25-hydroxyvitamin D <40 nmol/L) in Great Britain.

Taken from (Hypponen and Power, 2007).

1.5.8 Vitamin D determinants

The concentration of 25(OH)D concentration in the blood is depends strongly on sunlight exposure as well as dietary intake of vitamin D and on vitamin D supplementation. 25(OH)D concentration synthesis in the skin depends on latitude and season and also on other factors such as:

i. Adiposity

   Recent evidence suggests that obese individuals have lower circulating 25(OH)D concentrations than age and sunlight exposure-matched normal weight persons (Kevin McKinney, 2008; Ardawi *et al.*, 2012; Samuel and Borrell, 2013; Walsh *et al.*, 2016). This inverse relationship between vitamin D status and adiposity has been observed in multiple countries and in different age groups (Alyahya, 2017; Greene-Finestone *et al*., 2017). The lower 25(OH)D concentrations in obese people may be explained by adipose tissue sequestration and/ or volumetric dilution of 25(OH)D (Blum *et al.*, 2008; Drincic *et al.*, 2012) and/or negative feedback from higher circulating concentration of 25(OH)D (Pourshahidi, 2015).

ii. Skin pigmentation

Darker skin contains more melanin in the epidermal layer, which provides greater protection against ultraviolet radiation but reduces the skin's ability to produce vitamin D from sunlight (Bonilla *et al.*, 2014). Because of this protective effect, people with darker skin require more UVB exposure than those with lighter skin colors (Norman and Powell, 2014). The efficiency of cutaneous 25(OH)D concentration synthesis is lower in people with dark skin, as the high concentration of melanin absorbs and scatters the UVB rays, resulting in less efficient synthesis of 7-DHC to previtamin D_3 (SACN, 2016).

iii. Use of sunscreen and clothes style

Sunscreen prevents sunburn and skin cancer by blocking UVB light. However concerns have been raised that the application of sunscreen may prevent 25(OH)D concentration synthesis in the skin (SACN, 2016). The evidence supporting this concern is very limited and the published studies are insufficient to draw a concrete conclusion on the effects of sunscreen on 25(OH)D concentration synthesis (Springbett *et al.,* 2010). Clothing styles that prevent skin exposure to direct sunlight - may block the sun's rays, resulting in reduced 25(OH)D concentration synthesis (Mithal *et al.*, 2009; Nemerovski *et al.*, 2009; Bassil *et al.*, 2013).

iv. Indoor environments and institutionalized older persons

The elderly are especially at risk of 25(OH)D concentration deficiency as they are more likely than younger people to stay indoors, to use sunscreen (Gloth, 1995; Janssen *et al.*, 2002), to cover more of the skin with clothing whilst outside and to consume diets with less vitamin D (Gillespie *et al.*, 2012). The elderly cannot synthesize 25(OH)D concentration in the skin when exposed to the sun as well as when younger due to atrophic skin alterations with greater availability of the precursor 7-DHC (Hill *et al.*, 2016). Another factor that may contribute to age-related increased risk of vitamin D deficiency is reduced 1,25(OH)D synthesis due to lower renal function with ageing (Mosekilde, 2005). As renal function declines with age, the activity of the renal enzyme 1-α hydroxylase that converts 25(OH)D into 1,25(OH)D falls (Gallagher, 2013). Vitamin D receptor (VDR), as its name implies, function as the receptor for vitamin D, which decreased with advancing age. This condition can worsen the problems and put

the institutionalized elderly at a very high risk of low 25(OH)D concentration status (Mosekilde, 2005) in the absence of supplementation with vitamin D.

v. Air pollution

Carbon particulates in the air from the burning of fossil fuels, wood, and other materials scatter, and ozone absorbs UVB rays which may reduce dermal synthesis of 25(OH)D (Mithal *et al.*, 2009). A study done among 200, free-living, housewives, aged between 20 to 55 years, from Tehran (high polluted area) and Ghazvin (low polluted area) found that living in a polluted area plays a significant independent role in the aetiology of vitamin D deficiency (Hosseinpanah *et al.*, 2010). Another study in Belgian postmenopausal women also confirmed the positive correlation between air pollution and 25(OH)D deficiency (Manicourt and Devogelaer, 2008).

vi. Pregnancy and lactation

Exclusively breast fed infants are at risk of insufficiency of 25(OH)D concentration, because human milk provides approximately 25 IU of vitamin D per liter, which is not enough for an infant if it is the sole source of vitamin D (Oberhelman *et al.*, 2013). Most cases of rickets occur in exclusively breastfed infants who lack sunshine exposure and are not supplemented with vitamin D (Majid Molla *et al.*, 2000; Dawodu and Wagner, 2007). Vitamin D supply in breastmilk is reduced if the vitamin D status of the mother is low, the baby has darker pigmentation, they are living at higher latitudes, and in the winter season, so it is suggested that exclusively breastfed infants receive daily supplements of 400 IU of vitamin D when sunshine exposure is low or maternal 25(OH)D concentration status is inadequate (Holick, 1998; Pettifor, 2004; Wagner and Greer, 2008).

vii. Liver and Kidney Disease

As both liver and kidney are involved in the conversion of 25(OH)D concentration into its active form; i.e. the liver produces 25(OH)D, and kidney produced metabolically active 1,25(OH)D, the impairment of either of these two organs may result in vitamin D deficiency due to impaired synthesis. (Nair, 2010; Putz-Bankuti *et al.*, 2012).

1.6 Cardiovascular effects of vitamin D

Classically, vitamin D is important for calcium and phosphorus homeostasis which is essential for bone mineralisation and related musculoskeletal outcomes (Jones et al., 1998). However, the expression of the vitamin D receptor in multiple tissues suggests other potential functions of vitamin D in non-musculoskeletal health outcomes. There is growing evidence that vitamin D may plays role in determining risk of cardio-metabolic outcomes, particularly metabolic syndrome (MetS), type 2 diabetes mellitus, and systemic hypertension (Lavie *et al.*, 2013) and that adequate 25(OH)D concentration status help to prevent CVD (Holick, 2008).

A systematic review by Pittas et al (2010) reported that in longitudinal observational studies, there is a significant negative relationship between 25(OH)D concentrations and risk of major cardiovascular events and related chronic diseases, including hypertension, myocardial infarction (MI), stroke and type 2 diabetes. In contrast, in randomized controlled trials, there was no significant effect of vitamin D supplementation on blood pressure, glycaemic, or cardiovascular outcomes including MI, stroke and other cardiac and cerebrovascular outcomes (Pittas *et al.*, 2010). In a further systematic review, Elamin et al (2011) reported that vitamin D supplementation had no significant effect on death (RR, 0.96; 95% CI, 0.93, 1.00; P=0.08; I^2 =0%), stroke (RR,1.05; 95% CI, 0.88, 1.25; P=0.59; I^2 = 15%), MI (RR, 1.02; 95% CI, 0.88, 1.25; P = 0.59; I^2=15%) , lipid fractions, blood pressure, and blood glucose concentration in randomized controlled trials (Elamin *et al.*, 2011).

i. Vitamin D and EF

There are several possible mechanisms by which higher 25(OH)D concentration status could improve EF. 25(OH)D concentration is involved in the regulation of endothelial cell-dependent vasodilation. The effect may be mediated by the link of 25(OH)D concentration with renin angiotensin-aldosterone system (RAAS), a hormonal system that regulates blood pressure and fluid balance **(Figure 1.15)**. Elevated RAAS activity can contribute to hypertension and cardiovascular risk (Vaidya and Forman, 2012). Deficiency of 25(OH)D concentration predisposes to the up-regulation RAAS, hypertrophy of smooth muscle and a pro-inflammatory state which can increase the risk of hypertension and left ventricle hypertrophy (Wu *et al.*, 2010).

In addition, through its role in determining calcium homeostasis, 25(OH)D concentration may affect vascular smooth muscle cell function and, specifically the release of inflammatory cytokines such as such as IL-6, TNF-α and IFNγ which may influence vascular contractility (Polly and Tan, 2014) . Initial evidence suggested that VDR knockout mice are characterized by lower NO availability due to reduced expression of eNOS, leading to endothelial dysfunction, independent of changes in the renin-angiotensin system (Andrukhova *et al.*, 2014).

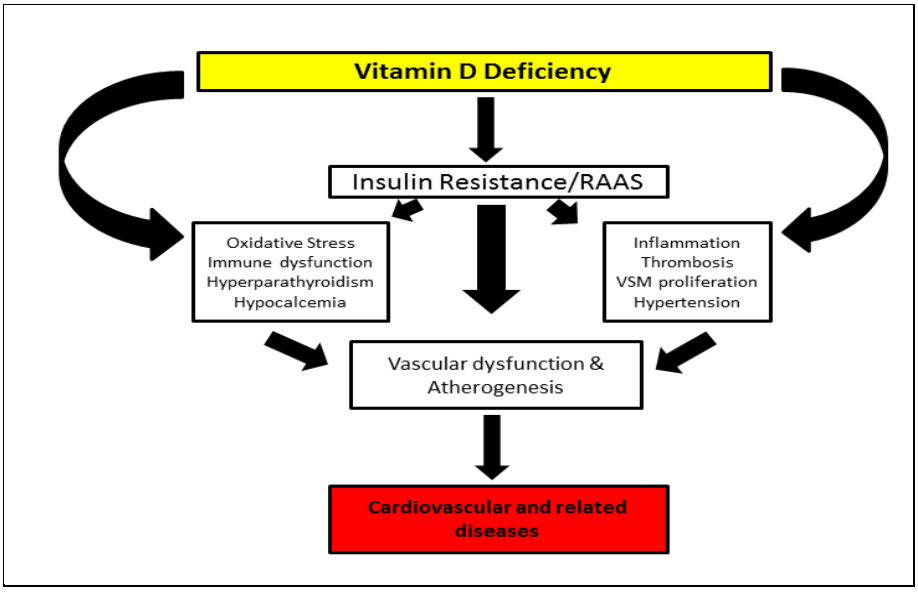


Figure 1. 15: Conceptual model of major pathways through which vitamin D deficiency may lead to increased CVD risk

(Artaza et al., 2009)

The VDR activates a wide variety of genes including vascular endothelial growth factor (VEGF) which in turn, promotes NO synthesis by endothelial cells (Ahmed *et al.*, 2013) **(Figure 1.16)**. Vascular smooth muscle and endothelial cells express VDR as well as 1α-hydroxylase (Somjen *et al.*, 2000) which enables autocrine production of 1,25(OH) D. This $1,25(OH)_2D$ may act locally to modulate the effects of inflammatory cytokines on the vasculature, such as decreasing endothelial adhesion molecules, increasing NO production (Molinari *et al.*, 2011) and reducing platelet aggregation (Aihara *et al.*, 2006). Despite this mechanistic evidence suggesting a role for 25(OH)D concentration in EF, a recent systematic review and meta-analysis found no consistent evidence that vitamin D supplementation in humans improves measures of arterial stiffness (Rodríguez *et al.*, 2016)

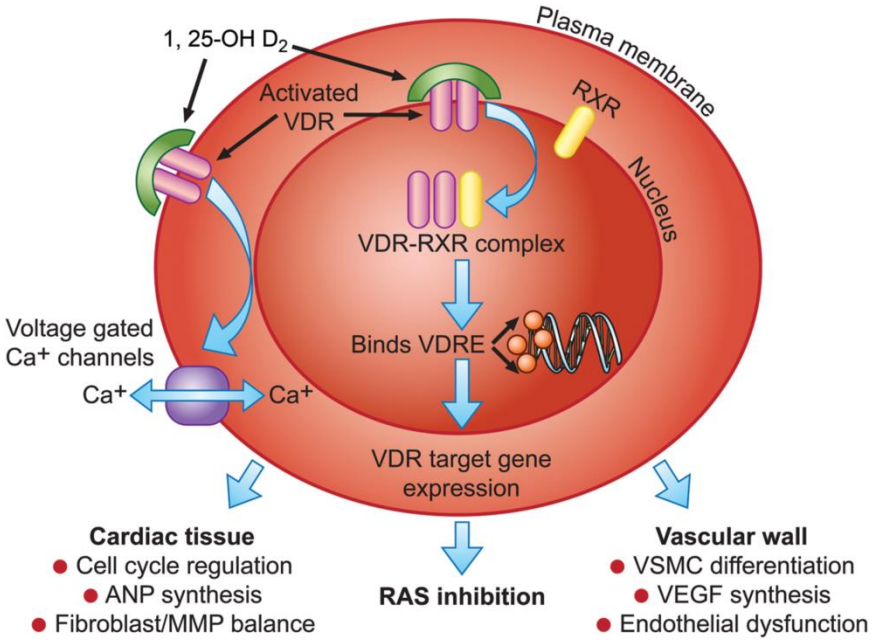


Figure 1. 16: Mechanisms by which vitamin D deficiency may increase cardiovascular disease risk.

Potential effects of vitamin D metabolism on the cardiovascular system are divergent, but share common initial steps of nuclear and plasma membrane VDR activation. VDR, vitamin D receptor; 1, 25-OH D2, 1, 25-dihydroxyvitamin D; RXR, retinoid-X receptor; Ca+, calcium cation; ANP, atrial natriuretic peptide; MMP, matrix metalloproteinases; VDRE, vitamin D response elements (promoter region of target genes); RAS, renin–angiotensin system; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells. Taken from (Al Mheid et al., 2013).

ii. Vitamin D and insulin sensitivity

Vitamin D status has been linked with the regulation of insulin secretion by the pancreatic beta cells. Deficiency of 25(OH)D concentration predisposes to insulin resistance and increased risk of type 2 diabetes (Lavie *et al.*, 2013), as calcitriol, via the VDR, regulates genes involved in insulin production in the pancreas (Lee *et al.*, 2008).

In addition, the 1- α – hydroxylase in pancreatic β-cells catalyses the conversion of 25(OH)D to 1,25$(OH)_2$D which activates transcription of the insulin receptor gene (Maestro *et al.*, 2002), and enhances insulin mediated glucose transport in vitro (Maestro *et al.*, 2000). 25(OH)D concentration has a close mechanistic link with the insulin signalling pathway **(Figure 1.17)**. Since insulin secretion is a calcium-dependent process, 25(OH)D deficiency may affect insulin secretion and sensitivity via alteration of extracellular and intracellular β-cell calcium pools and so interfere with normal insulin release (Nagpal *et al.*, 2005). Sustained elevation of

intracellular calcium concentration may inhibit insulin-target cells from sensing the brisk intracellular calcium fluxes necessary for insulin action, such as glucose transport (Worrall and Olefsky, 2002).

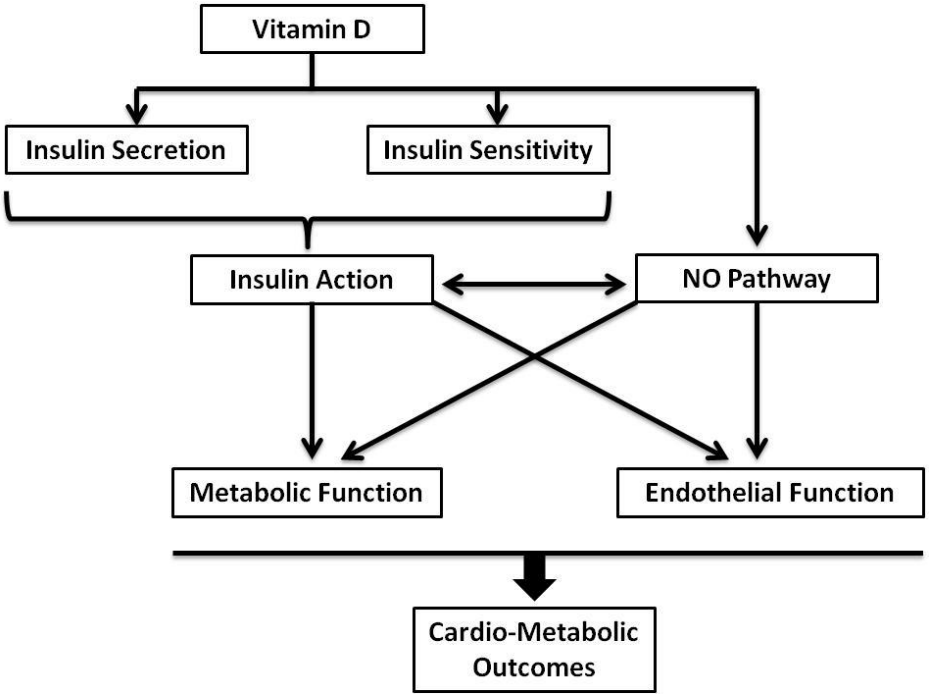


Figure 1. 17: Mechanistic link between vitamin D, insulin and NO pathway

1.7 Hypothesis, Aims and Objectives

Hypothesis

The hypothesis of this PhD programme was based on assumption that 25(OH)D concentration is involved in the regulation of EF via NO production. It is well established that NO initiates and maintains vasodilation in the endothelium, and 25(OH)D concentration may have an important role in regulating eNOS activity, which enhances the availability of NO.

Vitamin D is involved in vascular smooth muscle (VSM) function by modulating the regulation of growth and proliferation of VSM cells. Fat mass and ageing also modulate these physiological processes. Individuals with excess body weight tend to have lower vitamin D concentrations probably due to the sequestration of 25(OH)D in adipose tissue and/or volumetric dilution of 25(OH)D. Ageing is associated with decreased NO generation mediated by the reduction of the eNOS activity. Lower NO availability may increase the likelihood of endothelial cell senescence which may make the endothelium less responsive to hemodynamic

shear stress. As a consequences, as vessels age, blood flow may be reduced as resulted by less infective heart function (North and Sinclair, 2012).

It is postulated that 25(OH)D concentration status is positively associated with EF and with biomarkers of NO activity. In addition, fat mass is hypothesized to be a significant modulator of this association and it is anticipated that there will be stronger associations in lean compared with obese individuals. Similarly, ageing is predicted to be a significant modulating factor in the relationships between 25(OH)D concentration, EF and NO production and it is anticipated that stronger associations will be observed in older compared to younger individuals.

Aims

The aims of this PhD project were to investigate whether 25(OH)D status was a significant predictor of EF and *in vivo* whole body NO production, EF and cardiovascular biomarkers in two separate cohorts of individuals:

Project 1: Overweight and obese, post-menopausal women.

Project 2: Young and old, healthy male and female individuals to explore the association between age, whole-body NO production and vascular health in both men and women.

Objectives

The specific objectives of this PhD programme were to:

1. Conduct a systematic review of the effects of vitamin D supplementation on EF in randomized clinical trials (RCTs).
2. Test the accuracy of different methods for measuring nitrate, nitrite and 25(OH)D concentration in biological samples.
3. Test whether deproteination of plasma samples influences estimates of the plasma concentration of nitrate and nitrite using chemiluminescence.
4. Compare the estimates of circulating 25(OH)D concentrations using a conventional method (assays on plasma collected by venepuncture) and using a dry blood spot (DBS) method.
5. Conduct a cross-sectional study to evaluate the association of 25(OH)D concentration with biomarkers of EF in obese postmenopausal women.
6. Explore the association between ageing, 25(OH)D concentration and whole-body NO production.

Chapter 2. Effects of Vitamin D Supplementation on Endothelial Function: An Updated Systematic Review and Meta-Analysis

2.1 Introduction

Cardiovascular diseases (CVDs) are a major public health concern and contribute >30% of overall mortality worldwide (Dahlof, 2010). The pathogenesis of CVDs is multifactorial, and a critical step in the onset and advancement of CVDs is the formation of atherosclerotic lesions (Hansson, 2005). One of the earliest stages of the atherosclerosis process is the impairment of endothelial function (EF) (Higashi *et al.*, 2009).

The pathophysiology of endothelial dysfunction is complex and involves multiple mechanisms including over-production of reactive oxidative species (ROS), inflammatory cytokines and pro-atherogenic lipoproteins together with an imbalance between vasodilating and vasoconstricting molecules such as nitric oxide (NO), prostacyclin, Endothelium-Derived Hyperpolarizing Factor (EDHF), endothelin-1 (ET-1), angiotensin II (AngII), and thromboxane A2. Impairment of vasodilatation may be due to reduced availability of NO, which is produced by the endothelial cells and which is involved in multiple physiological processes including vasodilation, inflammation and platelet aggregation (Endemann and Schiffrin, 2004).

Vitamin D is a pro-hormone which plays well-established roles in the regulation of calcium homeostasis and in bone remodelling (Lips, 2006). However, vitamin D is also essential for several non-musculoskeletal functions including regulation of vascular tone, gluco-insular homeostasis and immunity (Lips, 2006). Vitamin D receptors (VDRs) are expressed in several tissues including endothelial cells, vascular smooth muscle cells and cardiomyocytes (Brandenburg *et al.*, 2012) so providing a plausible basis for a role of vitamin D in cardiovascular function. In addition, the active form of vitamin D (1α,25-dihydroxyvitamin D_3, $1,25(OH)_2D_3$) is a direct transcriptional regulator of endothelial NO synthase (Dalan *et al.*, 2014). A study has shown that VDR mutant mice have lower NO availability due to lower circulating of NO concentrations, leading to endothelial dysfunction, increased arterial stiffness, increased aortic impedance, structural remodelling of the aorta and impaired systolic and diastolic heart function (Andrukhova *et al.*, 2014). However, observational studies evaluating the association of vitamin D with CVD risk have reported mixed results. A

significant inverse relationship between vitamin D status, as assessed by circulation of serum 25-hydroxy vitamin D (25(OH)D) and risk of major cardiovascular events and chronic diseases such as myocardial infarction (MI), stroke, hypertension and type 2 diabetes has been reported (Liu *et al.*, 2005; Pittas *et al.*, 2006; Knekt *et al.*, 2008), but this has not been confirmed in other cohorts (Bajaj *et al.*, 2014; Sollid *et al.*, 2014). These discrepant results may be ascribed to the differences in study designs and to heterogeneity in the phenotypic characteristics of each study or of study participants including (1) duration of follow up, (2) cut-off values for the definition of deficient vitamin D status, (3) diagnostic criteria for the identification and classification of cardiovascular outcomes, (4) confounding factors (i.e., diet, sun exposure, seasonality, physical activity) and (5) health status of the participants in the cohorts (Pittas *et al.*, 2010).

Randomised controlled trials (RCTs) examining the effects of vitamin D supplementation on EF have also reported contradictory results; whilst some studies have reported improvement in EF (Sugden *et al.*, 2008; Harris *et al.*, 2011a; Breslavsky *et al.*, 2013) others have observed no effect of vitamin D supplementation (Witham *et al.*, 2010; Gepner *et al.*, 2012; Larsen *et al.*, 2012; Longenecker *et al.*, 2012; Marckmann *et al.*, 2012; Sokol *et al.*, 2012; Stricker *et al.*, 2012; Witham *et al.*, 2012; Beveridge and Witham, 2013; Hewitt *et al.*, 2013; Witham *et al.*, 2013b; Witham *et al.*, 2013c; Yiu *et al.*, 2013). A meta-analysis found a non-significant effect of vitamin D supplementation on changes in flow-mediated dilation measured by ultrasound after post-occlusion hyperaemia. The study showed that effects were greater in short studies (<16 weeks) and in participants with raised systolic and diastolic blood pressure (BP) (Stojanovic and Radenkovic, 2015).

The method selected for the assessment of EF in humans depends on the availability of resources and equipment, technical and research expertise and, most importantly, by the research question under investigation. The most commonly used methods to measure dynamic vascular responses are: 1) ultrasound to assess the increase in diameter of large arteries following post-occlusive hyperaemia, 2) plethysmography to asses changes in forearm blood flow during infusion of pharmacological agents targeting endothelial-related mechanisms (e.g. acetylcholine or sodium nitroprussiate) and 3) applanation tonometry by measuring pulse wave velocity (PWV) of peripheral arteries (Siervo *et al.*, 2011c).

In this chapter, the result of systematic review and meta-analysis of RCTs investigating the effect of supplemental vitamin D on EF were reported. The secondary aim of the study was to determine whether the effect size was modified by health status, study duration, dose, route of vitamin D administration, baseline vitamin D status and changes in 25(OH)D after supplementation, body mass index (BMI), age and type of vitamin D (vitamin D_2 or vitamin D_3).

2.2 Methods

The present systematic review was conducted according to the Cochrane guidelines (Higgins and Green, 2008) and it is reported according to PRISMA guidelines (Liberati *et al.*, 2009). This systematic review has been registered in the International Prospective Register of Systematic Reviews (PROSPERO) (Registration No: CRD42014009668).

2.2.1 Literature search

This chapter is an update of previous systematic review and meta-analysis performed by our group (Hussin *et al.*, 2017) which was published in the European Journal of Nutrition **(Appendix 2.1)**. Four databases (Medline, Embase, Scopus, and Cochrane Library) were used to search for articles from inception until March 2015. These databases were chosen as they are the ones used most widely for publications of research in medical, biomedical and life sciences and, therefore, are likely to contain reference to most relevant publications for this systematic review. In addition, they have easy-to-use search functions and allow the export of reference to Endnote (a widely used reference management system). Additionally, for the current update, eligible studies were searched in the articles from March 2015 until March 2017 to identify any additional relevant publications.

A manual search of reference lists of relevant reviews and articles included in the systematic review was performed. The search was conducted based on pre-defined search terms [Ergocalciferol OR Cholecalciferol OR vitamin D OR Vitamin D_2 OR vitamin D_3 OR 25(OH)D] And [Endotheli* OR Endotheli* dysfunction OR FMD or Hyperaemia OR Plethysmography OR Flow mediated OR Endothelial-dependent OR Vasomotor or Vasoacti* OR Blood flow OR Brachial OR Vasodilat* OR Dilat* OR Vascular resistance OR Pulse Wave OR Augmentation index OR Arterial stiffness OR Digital volume pulse OR Pulse amplitude tonometry OR Arterial compliance].

2.2.2 Study selection

The following criteria were applied to identify articles to be included in this systematic review and meta-analysis: (1) RCTs; (2) studies involving adults aged 18 years or more, and no exclusion criteria were applied for health status, smoking history or body size; (3) vitamin D administered alone i.e. not combined with other drugs or nutritional interventions; studies were not excluded on the basis of the dose, duration of follow up, route of administration of vitamin D or type of administration (i.e. tablet, capsule, solution or as fortified food) and type of assay used for the determination of 25(OH)D concentrations; (4) studies reporting changes in EF measured by ultrasound, venous-occlusion phlethysmography, photo phlethysmography, PWV, pulse amplitude tonometry, laser Doppler flowmetry; (5) no language or time restrictions were applied in searching the databases.

Two investigators (AMH and MS) independently screened the titles and abstracts of the articles to evaluate eligibility for inclusion. If consensus was reached, articles were either excluded or moved to the next stage (full text). If consensus was not reached, the articles were moved to the full-text stage. The full texts of the selected articles were appraised critically to determine eligibility for inclusion in the systematic review. Disagreements were resolved by discussion between the investigators until consensus was reached.

2.2.3 Data extraction and quality assessment

The following information was extracted from the eligible articles: (1) authors, journal details and year of publication; (2) participants (total number, male/female ratio, age, health status); (3) study characteristics (country, design, inclusion/exclusion criteria, description of measurement protocols; (4) vitamin D intervention (type, formulation, dose, duration of follow up, route of administration); (5) EF measurement (instrument, position, duration of cuffing) and (6) circulating concentrations of vitamin D before and after intervention.

In addition, the modified Jadad score was adopted to assess the risk of bias of the included studies; possible scores ranged from 0 to 5 and a score of ≤ 3 indicates high risk while a score of > 3 indicates low risk of bias (Crowther *et al.*, 2010).

2.2.4 Statistical analysis

Serum concentrations of 25(OH)D given in ng/mL were converted to nmol/L (1 ng/mL=2.46 nmol/L)(*Dietary Reference Intakes for Calcium and Vitamin D*, 2011). Several methods were

used to assess EF in the included studies including flow mediated dilation (FMD), forearm blood flow (FBF), pulse wave analysis (PWA) and laser Doppler (LD) with the results obtained from these methods reported on different scales. Therefore, to allow comparison of effect sizes between studies, standardised mean differences (SMDs) were used as a summary statistic. SMD is estimated from the difference between the mean outcome values of the intervention and control groups divided by the pooled standard deviation (SD) of the outcome values; this converts the estimated effect to SD units. SMD of 0.2, 0.5 and 0.8 represent small, medium and large effect sizes, respectively (Cummings, 2011). In addition, different methods were frequently used in the same trial to assess EF, as shown in **Table 2.1**, and therefore this lack of independence of the EF measurement in each trial was taken into consideration in the derivation of the pooled effect size. Statistical analyses were performed by using Comprehensive meta-analysis software (version 2, Biostat, Englewood, New Jersey, USA). Data synthesis, including calculation of effect sizes with 95% confidence intervals, was accomplished by employing a random-effects model using inverse variance weighting. Forest plots were generated for graphical presentation of the effect of supplemental vitamin D on EF. For this purpose, the mean and SD of the EF measure before and after the intervention period (for both vitamin D intervention and control) were extracted and used in the analysis. For studies that reported changes in EF at two or more time-points (e.g. acute and chronic effects of vitamin D supplementation), the last EF measurement (chronic effect) was used in the meta-analysis. Data not provided in the main text or tables were extracted from the figures.

Subgroup analyses were undertaken to investigate the variables which may have influenced the effects of supplementation on EF. These factors included: health status, type (vitamin D_2 or D_3) and the frequency of administration (single dose, daily-weekly or monthly) of vitamin D supplementation. Random effect meta-regression analyses were used to determine whether participant baseline characteristics (age, BMI, systolic and diastolic blood pressure, baseline concentration of 25-OHD) influenced the effect of vitamin D supplementation (vitamin D_2 or D_3) on EF. Furthermore, meta-regression analyses were conducted to investigate the influence of other factors including vitamin D dose, baseline 25(OH)D, change in 25(OH)D concentration after supplementation, duration of interventions, sample size and quality score (Jadad score) on the effect of vitamin D supplementation on EF.

Heterogeneity between studies was evaluated using Cochrane Q statistics; $p > 0.1$ indicates significant heterogeneity. The I^2 test was also used to evaluate consistency between studies where a value <25% indicates low risk of heterogeneity, 25-75% indicates moderate risk of heterogeneity and >75% indicates high risk of heterogeneity (Higgins *et al.*, 2003). The evidence of publication bias was assessed by visual inspection of the funnel plots and by Egger's regression test (Egger *et al.*, 1997).

2.3. Results

2.3.1 Search results

The process of screening and selection of studies is summarised in **Figure 2.1**. The original primary search of the four databases produced 4159 articles after removal of duplicates. After title and abstract screening, 22 full-text papers were retrieved for further evaluation. Additionally, one study was found by manual searching references of the relevant reviews and studies. Examination of the full text of 23 articles yielded 16 studies which were eligible to be included in this systematic review and meta-analysis. One trial (Witham *et al.*, 2010) included two independent arms supplementing different vitamin D doses, which resulted in 17 independent interventions entered in the meta-analysis. The article describing that original analysis was published by (Hussin *et al.*, 2017). For the work reported in this chapter, the reviews were then extended to accommodate any more recently published papers and four additional articles (Dalan *et al.*, 2014; Pilz *et al.*, 2015; Witham *et al.*, 2015; Borgi *et al.*, 2017) published within the time frame (March 2015 to March 2017) were found and are included in this review. Therefore, the final number of articles included in this review is 20.

Table 2. 1: Summary of findings from 20 publications included in systematic review and meta-analysis

Author	Country	Health Status	Outcome	Sample Size	Male (N)	Age (years)	BMI (kg/m^2)	SBP/DBP (mmHg)	Vit D Dose (IU)	Duration (Frequency)	Formulation (Route)	Baseline Vit D (Assay)	Δ Vit D (nmol/L)	Jadad Score
Breslavsky et al. 2013 (Breslavsky *et al.*, 2013)	Israel	T2D	AI	47	22	67	29	153/74	1000	52w (D)	Capsule (Oral)	29 (IA)	17	3
Gepner et al. 2012 (Gepner *et al.*, 2012)	US	Healthy	PWV, FMD,AI	109	0	64	26	122/72	2500	16w (D)	Biscuits (Oral)	78 (LC-MS)	39	5
Harris et al. 2011 (Harris *et al.*, 2011a)	US	OW	FMD	45	21	29	30	123/74	60,000	16w (M)	Capsule (Oral)	36 (IA)	66	4
Hewitt et al. 2013 (Hewitt *et al.*, 2013)	Australia	CKD	PWV, FMD	60	29	60	29	131/76	50,000	8w (WK)	Solution (Oral)	42 (IA)	42	4
Larsen et al. 2012 (Larsen *et al.*, 2012)	Denmark	Ht	AI, PWV	130	35	60	28	131/77	3000	20w (D)	Tablet (Oral)	57 (IA)	52	5
Longenecker et al. 2012(Longenecker *et al.*, 2012)	US	HIV	FMD	45	35	47	27	118/80	4000	12w (D)	Capsule (Oral)	19 (IA)	12	5
Marckmann et al. 2012 (Marckmann *et al.*, 2012)	Denmark	CKD	PWV, FMD	52	39	71	25	135/72	40,000	8w (WK)	Capsule (Oral)	28 (LC-MS)	118	4
Sokol et al. 2012 (Sokol *et al.*, 2012)	US	CHD	RH-PAT	90	66	55	30	133/76	50,000	12w (WK)	Tablet (Oral)	84 (LC-MS)	67	3
Stricker et al. 2012 (Stricker *et al.*, 2012)	Switzerland	PAD	AI	62	38	73	27	136/74	100,000	4w (SD)	Solution (Oral)	41 (IA)	19	4
Sugden et al. 2008 (Sugden *et al.*, 2008)	UK	T2D	FMD	34	18	65	31	141/80	100,000	8w (SD)	Solution (Oral)	38 (IA)	23	3
Witham et al. 2010 (Witham *et al.*, 2010)	UK	T2D	FMD	61	41	G1:65 G2:63	G1:31 G2:32	G1:141/76 G2:128/72	G1:100000 G2:200000	16w (SD)	Solution (Oral)	G1: 46 (IA) G2: 43 (IA)	G1:28 G2:18	5
Witham et al. 2012 (Witham *et al.*, 2012)	UK	Stroke	FMD	58	42	66	27	129/72	100000	16w (SD)	Solution (Oral)	38 (IA)	12	3
Witham et al. 2013 (Beveridge and Witham, 2013)	UK-South Asian	Healthy	FMD, PWV, AI, LD-ION	50	0	41	27	121/78	100000	8w (SD)	Solution (Oral)	27 (IA)	10	5
Witham et al. 2013 (Witham *et al.*, 2013b)	UK	MI	RHI	75	52	64	27	128/72	100000	24w (2M)	Solution (Oral)	47 (IA)	13	4
Witham et al.2013 (Witham *et al.*, 2013c)	UK	ISH	FMD, PWV	159	82	77	28	163/78	100000	52w (3M)	Solution (Oral)	45 (IA)	25	5
Yiu et al. 2012 (Yiu *et al.*, 2013)	China	T2D	FMD, PWV	100	66	50	25	146/81	5000	12w (D)	Tablet (Oral)	54 (IA)	92	3
Dalan et al. 2016 (Dalan *et al.*, 2016a)	Singapore	T2D	RHI, AI	61	33	54	28	139/77	1000	16w (D)	Tablet (Oral)	43(IA)	35	5
Pilz et al 2015 (Pilz *et al.*, 2015)	Austria	Ht	PWV	200	106	60	30	143/86	2800	8w (D)	Oily drops (Oral)	52(IA)	28	5
Witham et al 2015 (Witham *et al.*, 2015)	UK	CFS/ME	PWV,FMD	50	12	49	29	128/78	100,000	24w (2M)	Oily drops (Oral)	46(IA)	18	3
Borgi et al 2016 (Borgi *et al.*, 2017)	US	OW, obese	EDV	93	30	37	34	118/78	50000	8w (WK)	Tablet (Oral)	37(IA)	71	3

N, number of subjects; T2D, Type 2 Diabetes; OW, overweight; CKD, Chronic Kidney Disease; Ht, Hypertension; HIV, Human Immunodeficiency Virus; CHD, Coronary Heart Disease; PAD, Peripheral Arterial Disease; MI, Myocardial Infarction; ISH, Isolated systolic Hypertension ; CFS/ME, Chronic fatigue syndrome/<myalgic encephalomyelitis; AI; Augmentation Index; PWV, Pulse Wave Velocity; FMD, Flow Mediated Dilatation; RH-PAT, Reactive Hyperaemia Peripheral Arterial Tonometry; LD-ION: Laser Doppler Iontophoresis; RHI, Reactive Hyperaemia Index; EDV; Endothelial-dependent vasodilation; Group 1: 100 000 IU , Group 2: 200 000 IU; IU, International Unit; D, daily; w, weeks; WK, weekly; M, monthly; SD, single dose; 2M, every 2 months; 3M, every 3 months. Δ= changes in vitamin D concentrations after supplementation.; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; IA, Immunoassay; LC-MS, Liquid chromatography–mass spectrometry

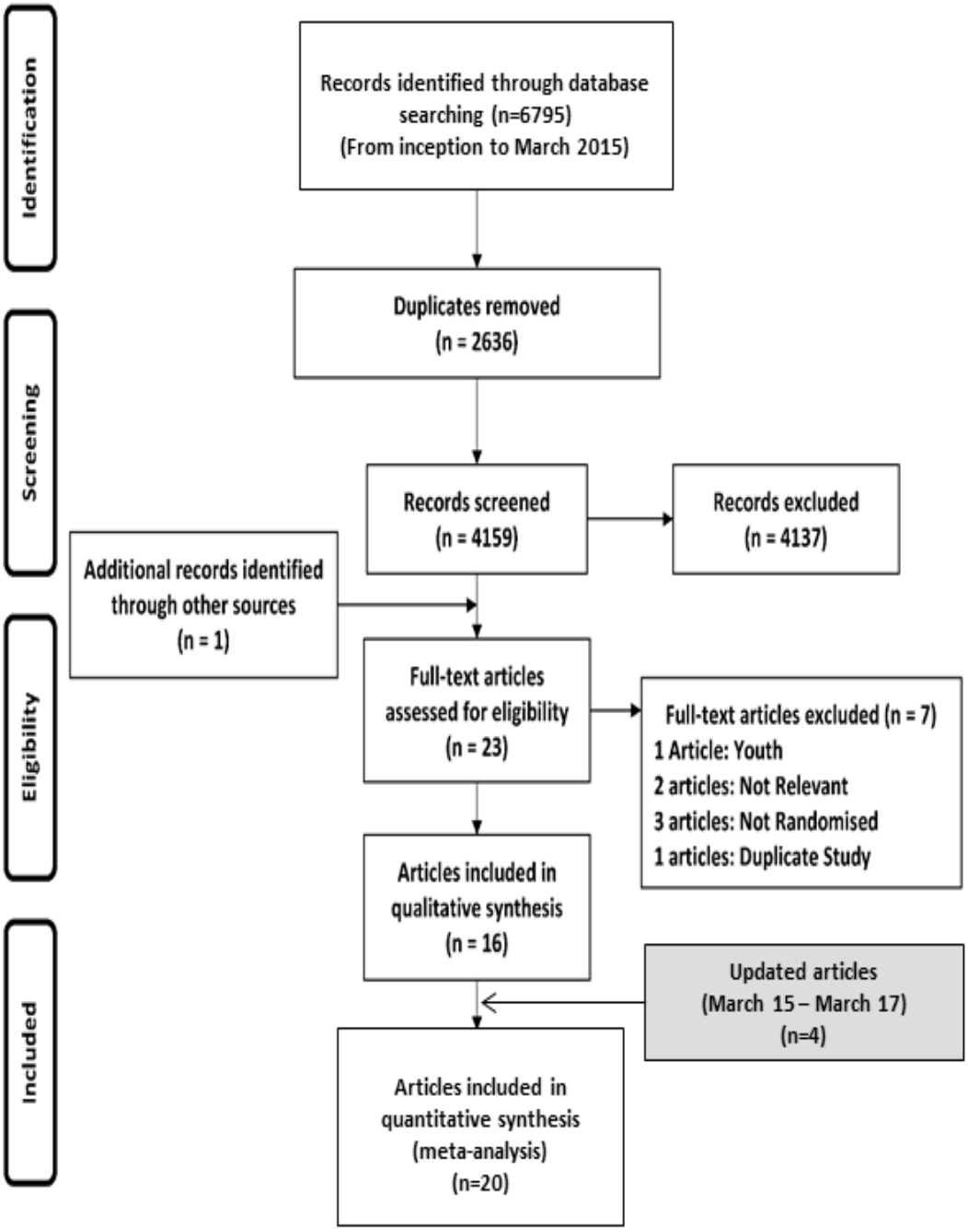


Figure 2. 1: Flow diagram of the selection process of the randomised clinical trials included in the systematic review and meta-analysis.

2.3.2 Studies characteristics

The total number of participants from the 20 studies included in this systematic review was 1581 (814 females; 767 males) with median of 79 (range 34 -200) participants per study. The median age was 57.6 (range 37-77) years. All RCTs included in the meta-analysis were parallel, double-blind, placebo-controlled trials. The duration of the trials ranged from 4 weeks to 52 weeks **(Table 2.1)**.

Four studies investigated the effect of vitamin D in healthy participants (Harris *et al.*, 2011a; Witham *et al.*, 2013a; Gepner *et al.*, 2015; Borgi *et al.*, 2017), two studies were conducted in patients with chronic kidney disease (Marckmann *et al.*, 2012; Hewitt *et al.*, 2013), five studies in diabetics (Sugden *et al.*, 2008; Witham *et al.*, 2010; Breslavsky *et al.*, 2013; Yiu *et al.*, 2013; Dalan *et al.*, 2016b), seven studies in patients with CVDs (Larsen *et al.*, 2012; Sokol *et al.*, 2012; Stricker *et al.*, 2012; Witham *et al.*, 2012; Witham *et al.*, 2013b; Witham *et al.*, 2013c; Pilz *et al.*, 2015), one study in patients with chronic fatigue syndrome/myalgic encephalomyelitis (Witham *et al.*, 2015) and one study in patients with HIV (Longenecker *et al.*, 2012). In all trials, participants were supplemented orally with vitamin D. Trials however utilised different forms of supplementation including tablets (Larsen *et al.*, 2012; Sokol *et al.*, 2012; Yiu *et al.*, 2013; Dalan *et al.*, 2016b; Borgi *et al.*, 2017), solution (Sugden *et al.*, 2008; Witham *et al.*, 2010; Stricker *et al.*, 2012; Witham *et al.*, 2012; Hewitt *et al.*, 2013; Witham *et al.*, 2013a; Witham *et al.*, 2013b), capsules (Harris *et al.*, 2011a; Longenecker *et al.*, 2012; Marckmann *et al.*, 2012; Breslavsky *et al.*, 2013), fortified biscuits (Gepner *et al.*, 2012) and oil drops (Pilz *et al.*, 2015; Witham *et al.*, 2015). The majority of the trials utilised vitamin D_3 with daily doses varying from 1000 IU/day (Breslavsky *et al.*, 2013; Dalan *et al.*, 2016a) to 5000 IU/day (Yiu *et al.*, 2013).

Several methods were used to assess EF in the included trials. The most commonly used methods were flow-mediated dilation (FMD) (Sugden *et al.*, 2008; Witham *et al.*, 2010; Harris *et al.*, 2011a; Gepner *et al.*, 2012; Longenecker *et al.*, 2012; Marckmann *et al.*, 2012; Hewitt *et al.*, 2013; Witham *et al.*, 2013c; Yiu *et al.*, 2013), pulse wave velocity (PWV) (Gepner *et al.*, 2012; Larsen *et al.*, 2012; Marckmann *et al.*, 2012; Hewitt *et al.*, 2013; Witham *et al.*, 2013a; Witham *et al.*, 2013c; Yiu *et al.*, 2013; Pilz *et al.*, 2015) and augmentation index (AIx) (Gepner *et al.*, 2012; Larsen *et al.*, 2012; Stricker *et al.*, 2012; Breslavsky *et al.*, 2013; Gepner *et al.*, 2015; Dalan *et al.*, 2016b). Other methods include laser Doppler flowmetry (Witham *et*

al., 2013a), digital volume pulse (Witham *et al.*, 2013b) and endothelial-dependent vasodilation (Borgi *et al.*, 2017) **(Table 2.1)**.

2.3.3 Qualitative analysis

Three of the studies included in the present systematic review reported a significant improvement in EF in response to vitamin D administration (Sugden *et al.*, 2008; Harris *et al.*, 2011b; Breslavsky *et al.*, 2013) whereas the other 17 studies reported no effect of supplementation (Witham *et al.*, 2010; Gepner *et al.*, 2012; Larsen *et al.*, 2012; Longenecker *et al.*, 2012; Marckmann *et al.*, 2012; Sokol *et al.*, 2012; Stricker *et al.*, 2012; Witham *et al.*, 2012; Hewitt *et al.*, 2013; Witham *et al.*, 2013b; Witham *et al.*, 2013c; Yiu *et al.*, 2013; Gepner *et al.*, 2015; Pilz *et al.*, 2015; Witham *et al.*, 2015; Dalan *et al.*, 2016b; Borgi *et al.*, 2017). Fifteen studies described the methods of randomisation (Witham *et al.*, 2010; Gepner *et al.*, 2012; Larsen *et al.*, 2012; Longenecker *et al.*, 2012; Marckmann *et al.*, 2012; Sokol *et al.*, 2012; Beveridge and Witham, 2013; Hewitt *et al.*, 2013; Witham *et al.*, 2013b; Yiu *et al.*, 2013; Gepner *et al.*, 2015; Pilz *et al.*, 2015; Witham *et al.*, 2015; Dalan *et al.*, 2016b; Borgi *et al.*, 2017), and five studies stated the methods of allocation concealment (Witham *et al.*, 2010; Larsen *et al.*, 2012; Longenecker *et al.*, 2012; Beveridge and Witham, 2013; Witham *et al.*, 2013b). The drug history of the participants was reported by all except three studies (Harris *et al.*, 2011a; Beveridge and Witham, 2013; Breslavsky *et al.*, 2013). With the exception of two studies (Harris *et al.*, 2011a; Hewitt *et al.*, 2013), all other studies reported, and described, participant dropout. The quality of the included studies ranged from 3 to 5 (Jadad score) and thirteen studies had a low risk of bias (Jadad score ≥4) **(Table 2.1).**

2.3.4 Meta-analysis

Meta-analysis of the 20 studies (1581 participants) showed that, overall, there was a non-significant trend for vitamin D supplementation improve EF (SMD: 0.10, 95% CI: -0.01, 0.20, p=0.08) **(Figure 2.2)**. There was no overall effect of supplemental vitamin D on post-occlusive vasodilation of the brachial artery (FMD %, N=11, +0.09 %, 95 % CI: -0.08, 0.27, p=0.30) **(Table 2.2)**.

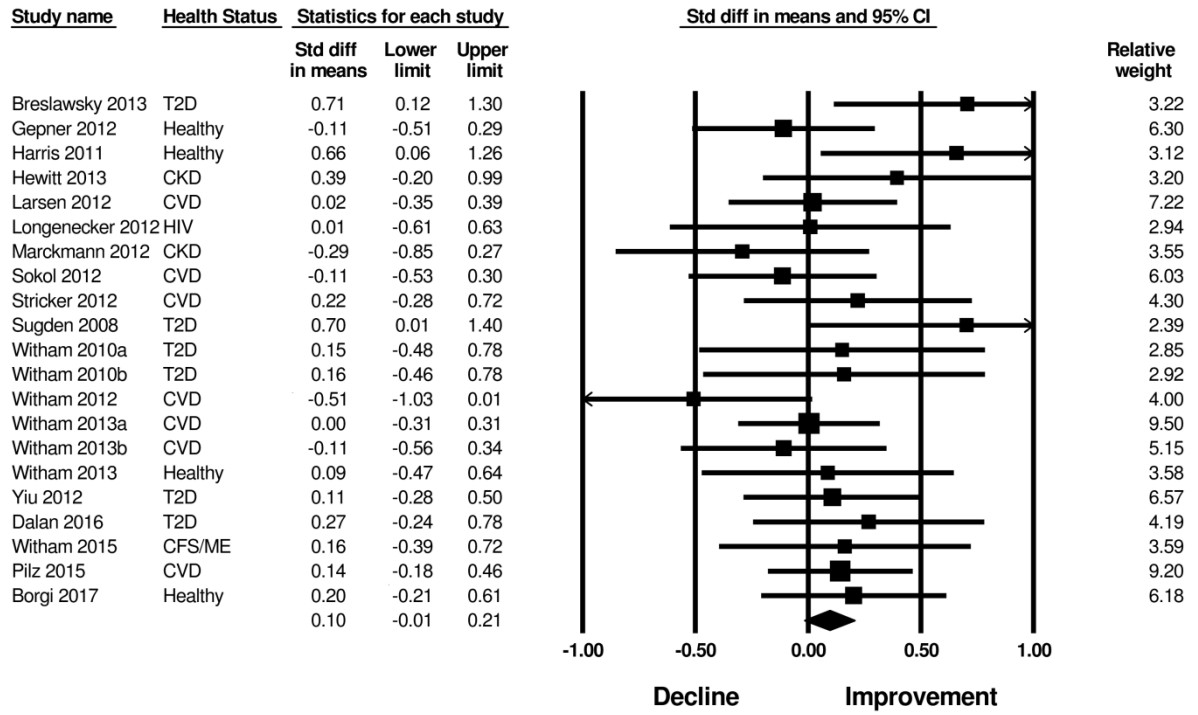


Figure 2. 2: Forest plot showing the effect of vitamin D supplementation on endothelial function.

T2D, type 2 diabetes; CVD, cardiovascular disease; CKD, chronic kidney disease; CFS/ME, Chronic Fatigue Syndrome/Myalgic Encephalomyelitis; HIV, Human Immunodeficiency Virus. Relative weight for a random model allows for small size studies contributing in a similar magnitude to the pooled estimate. The marker may be vary in size according to the weights assigned to the different studies. The pooled effect is represented using a diamond.

Heterogeneity between studies was not significant (Q=22.8, I^2=12.5%, P=0.29). However, subgroup analysis showed that vitamin D supplementation improved EF significantly in participants with type 2 diabetes (N=5, SMD: 0.24, 95 % CI: 0.06, 0.42, P=0.003) **(Table 2.3)**. This was also confirmed by the significant effect of vitamin D supplementation in type 2 diabetics on changes in FMD % (N=4, +0.28 %, 95 % CI: 0.00, 0.55, p=0.04 **(Table 2.2)**. The response of EF to vitamin D supplementation was not significantly modified by baseline health status of the participants, method of administration, baseline 25(OH)D concentrations or type of vitamin D **(Table 2.3)**. Meta-regression analyses demonstrated a significant effect of BMI (β: 0.04, SE: 0.01, P=0.03) and there was a trend of significant association of baseline diastolic blood pressure (β: 0.02, SE: 0.01, p=0.06) with the effect size on EF of vitamin D supplementation **(Table 2.4)**.

Table 2. 2: Effect of vitamin D supplementation on endothelial function measured by flow mediated dilation (FMD) of brachial artery in the total samples and in subjects with type 2 diabetes

	Number Studies	Point estimate (%)[a]	Standard error	±95%CI	P-value
Total Sample	11	0.09	0.09	-0.08 0.27	0.30
Type 2 Diabetes	4	0.28	0.13	0.00 0.55	0.04

[a]Point estimate is the post-occlusive percent dilation of brachial artery

Table 2. 3: Sensitivity analysis to evaluate the influence of health status, frequency of administration of vitamin D and type of vitamin D dose and baseline 25(OH)D concentration on the effect of vitamin D supplementation on endothelial function

Group	No of trials or subgroup	Effect size	95% CI	*p*	*p* between groups
Health status					
• Healthy	4	0.05	-0.10 0.21	0.47	
• HIV	1	0.00	-0.61 0.62	0.97	
• T2D	5	0.24	0.06 0.42	0.003	
• CKD	2	-0.07	-0.40 0.25	0.64	0.35
• CVD	7	-0.00	-0.12 0.11	0.95	
• CFS/ME	1	0.16	-0.23 0.55	0.41	
Frequency of Dose Administration					
• 1-2 months	2	0.16	-0.23 0.55	0.41	
• 1-3 month	5	0.08	-0.09 0.25	0.38	0.12
• Daily-Weekly	8	-0.02	-0.13 0.08	0.69	
• Single dose	5	0.10	-0.07 0.27	0.26	
Baseline 25-OHD concentration					
Normal (≥50nmol/L)	5	0.01	0.00 0.20	0.04	
Deficient (<50nmol/L)	15	-0.00	-0.12 0.12	0.98	0.12
Vitamin D type					
• D_2	3	-0.09	-0.33 0.14	0.42	0.12
• D_3	17	0.08	-0.00 0.16	0.05	

HIV, Human Immunodeficiency Virus; T2D, type 2 diabetes; CVD, cardiovascular disease; CKD, chronic kidney disease; CFS/ME, Chronic Fatigue Syndrome/ Myalgic Encephalomyelitis; D_2, ergocalciferol; D_3, cholecalciferol; 25(OH)D, 25 hydroxy vitamin D.

Table 2. 4: Meta-regression analysis to evaluate the association of potential modifiers of the effects of vitamin D supplementation on endothelial function

Covariates	Slope	SE	Q (df=1)	P Value
Baseline Systolic BP (mmHg)	0.002	0.003	0.35	0.55
Baseline Diastolic BP (mmHg)	0.02	0.01	3.38	**0.06**
Vitamin D concentration at baseline (nmol/L)	-0.003	0.002	3.12	0.07
Change in 25(OH)D concentration after supplementation (nmol/L)	-0.001	0.001	0.83	0.35
Study Duration (weeks)	0.001	0.003	0.01	0.90
Vitamin D Dose (IU)	0.0001	0.00001	0.00	0.96
Age (years)	-0.003	0.004	2.25	0.13
BMI (kg/m^2)	0.04	0.01	4.39	**0.03**
Study Sample Size (N)	-0.001	0.001	0.69	0.40
Jadad Score	-0.02	0.04	0.30	0.57

BP, blood pressure; BMI, body mass index; N, number of study participants; 25(OH)D, 25 hydroxy vitamin D.

BMI did not modify the association between type 2 diabetes and EF (N=8, β: 0.04, SE: 0.03, *p*=0.23) **(Appendix 2.2)** whereas lower baseline 25(OH)D concentrations were associated with larger effect sizes in type 2 diabetic participants (N=8, β: -0.02, SE: 0.01, *p*=0.04) **(Figure 2.3)**. The dose of vitamin D was not associated with significant changes in EF **(Table 2.4** and **Figure 2.4)**.

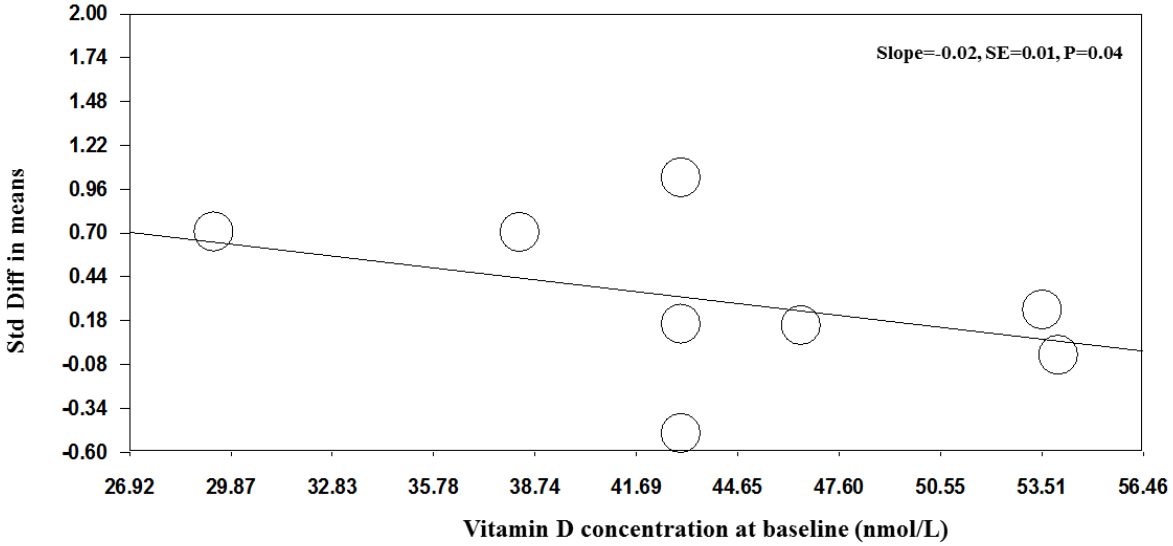


Figure 2. 3: Meta-regression analysis to evaluate the influence of baseline vitamin D concentration on the effects of vitamin D supplementation on endothelial function in type 2 diabetic patients

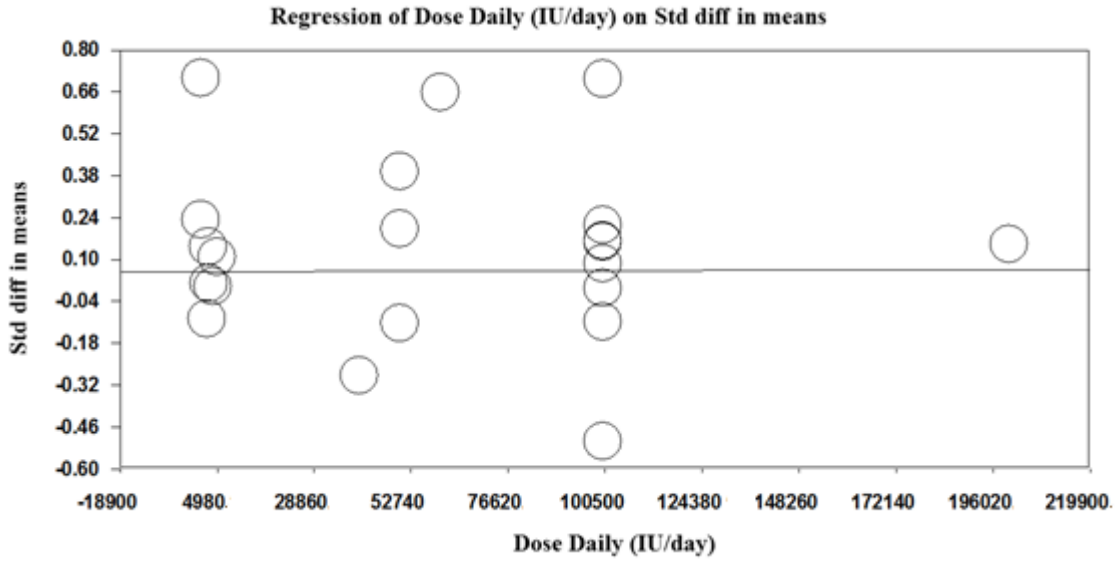


Figure 2. 4: Meta-regression analysis to evaluate the association between daily dose of vitamin D and the effects of vitamin D supplementation on endothelial function (P = 0.96).

2.3.5 Publication bias

Visual inspection of the funnel plot showed modest evidence of asymmetric distribution of the effect size **(Figure 2.5)**, which was confirmed formally by the lack of significance of the Egger's test (p=0.06).

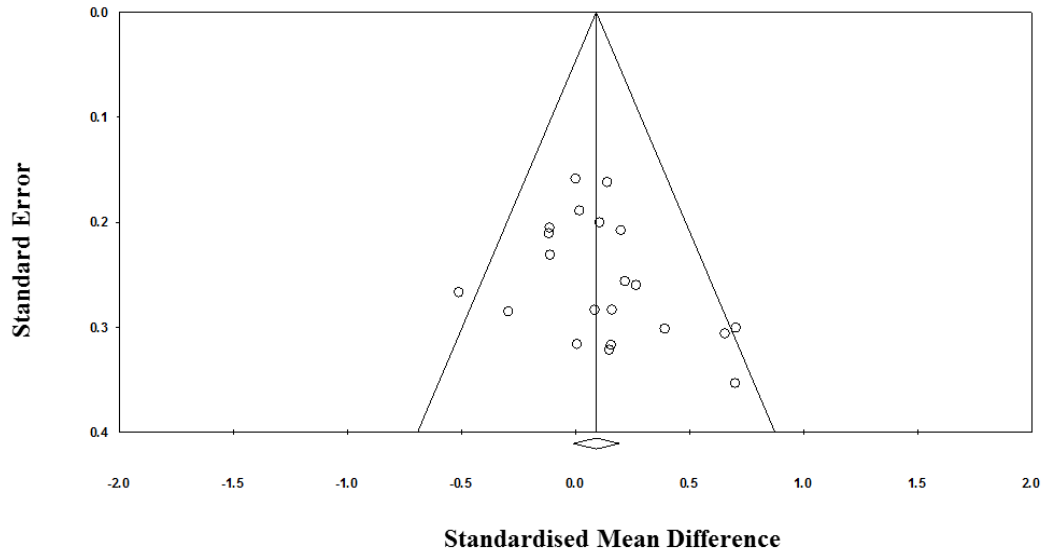


Figure 2. 5: Funnel plot examining the presence of publication bias in the meta-analysis of the effects of vitamin D supplementation on endothelial function.

2.4. Discussion

Overall, this meta-analysis demonstrated no effect of vitamin D supplementation on EF. In addition, changes in vitamin D concentration after supplementation were not associated with effects of vitamin D supplementation on EF. However, vitamin D supplementation resulted in a significant improvement in EF in patients with diabetes, and there was a significant, positive trend towards greater effects of vitamin D on EF with increasing baseline BMI and an almost significant association with baseline diastolic blood pressure.

Contrary to this meta-analysis, a recent systematic review and meta-analysis including 12 RCT conducted by (Mazidi *et al.*, 2017) reported a positive effect of vitamin D supplementation on endothelial function. In the latter analysis, flow mediated dilation alone was chosen as the method for measuring endothelial function. In addition, the RCT included in this review supplemented with both vitamin D_2 and D_3, and one study provided paricalcitol (an analogue of 1,25-dihydroxyergocalciferol, the active form of vitamin D_2) as the form of vitamin D supplementation in chronic kidney disease patients.

In contrast, the systematic review reported in this chapter was more inclusive and included studies regardless of the method used for measuring endothelial function. This resulted in the inclusion of articles using Augmentation Index, Pulse Wave Velocity, Flow Mediated Dilatation, Reactive Hyperaemia Peripheral Arterial Tonometry; Laser Doppler Iontophoresis, Reactive Hyperaemia Index and Endothelial-dependent vasodilation. The diversity of the methods for measuring endothelial function in this chapter might have contributed to the contrasts findings when compared with the systematic review and meta-analysis done by (Mazidi *et al.*, 2017).

Several putative mechanisms could explain the positive effects of vitamin D on EF in some population groups, particularly in those at higher cardiovascular risk. Vitamin D is involved in the regulation of endothelial cell-dependent vasodilation, which may be mediated by the effect of vitamin D metabolites on the renin angiotensin-aldosterone system, a hormonal system that regulates blood pressure and fluid balance. A low plasma 25(OH)D predisposes to up-regulation of the renin-angiotensin system, smooth muscle proliferation and favours a pro-inflammatory state, which can increase the risk of hypertension and left ventricle hypertrophy (Wu *et al.*, 2010). The improvement in EF through vitamin D supplementation could also be mediated by the local effects of vitamin D metabolites on calcium metabolism in vascular smooth muscle cells and on the release of inflammatory cytokines, which may affect vascular contractility (Kassi *et al.*, 2013). Vascular smooth muscle and endothelial cells express VDR as well as 1α-hydroxylase (Somjen *et al.*, 2000), allowing for autocrine production of $1,25(OH)_2D$, which may act at the local level to modulate the effects of inflammatory cytokines on the vasculature, such as decreasing endothelial adhesion molecules, increasing NO production (Molinari *et al.*, 2011) and reducing platelet aggregation (Aihara *et al.*, 2006). Binding of $1,25(OH)D_2$ and activation of the VDR induces the transcription of a wide range of genes including those coding for vascular endothelial growth factor, which in turn promotes NO synthesis by endothelial cells (Ahmed *et al.*, 2013). In addition, $1,25(OH)_2D$ is a direct regulator of endothelial NO synthase (Andrukhova *et al.*, 2014).

Vitamin D may also have beneficial effects on cardio-metabolic health in those with hypertension (Bednarski *et al.*, 2007; Almirall *et al.*, 2010; Cosenso-Martin and Vilela-Martin, 2011; Chai *et al.*, 2013), type 2 diabetes (Borissova *et al.*, 2003; Knekt *et al.*, 2008; Yiu *et al.*, 2013) and CVD (Grandi *et al.*, 2010; Nadir *et al.*, 2010; Al Mheid *et al.*, 2011). A meta-analysis of data from 21 prospective studied showed an inverse association between vitamin D status

and risk of type 2 diabetes (Song *et al.*, 2013). In addition, cardiovascular disease is the main cause of premature mortality and morbidity in patients with CKD (Marckmann *et al.*, 2012). These cardiovascular complications may be related to hypovitaminosis (Williams *et al.*, 2009), which may be linked to the inability of renal mass to convert 25(OH)D to the active form of vitamin D, 1, 25-dihydroxyvitamin (Ruggiero and Pacini, 2009). However, this analysis did not show a significant effect of vitamin D supplementation on EF in patients with CKD, which could be explained by several factors including the small number of studies (only two trials), the short duration of supplementation (8 weeks), the inadequacy of the vitamin D dose or the advanced stage of endothelial dysfunction. Alternatively, such vitamin D supplementation may be ineffective in improving EF in CKD patients.

In the present meta-analysis, vitamin D supplementation was observed to produce a significant improvement in vascular function in individuals with type 2 diabetes. While the small number of trials included in the analyses (n=5) calls for cautious and objective interpretation of the finding, this is supported by a robust mechanistic rationale. This apparent disease-specific effect may be explained by several mechanisms including the link between low 25(OH)D concentrations and (1) deterioration of β-cell function, (2) dysregulation of peripheral insulin signalling and (3) altered glucose disposal which are characteristic features in the pathogenesis of type 2 diabetes (Pittas *et al.*, 2007b; Knekt *et al.*, 2008; Pittas *et al.*, 2010). These effects appear to be supported by the greater effect of vitamin D supplementation on EF in type 2 diabetic patients with insufficient vitamin D status. Vitamin D receptors and 1-α-hydroxylase are expressed in pancreatic β-cells and therefore, an involvement of vitamin D in the regulation of insulin secretion may be expected (Borissova *et al.*, 2003; Talaei *et al.*, 2013; Al-Shoumer and Al-Essa, 2015). In turn, $1,25(OH)_2D$ activates transcription of the human insulin receptor gene, stimulates expression of the insulin receptor (Maestro *et al.*, 2002) and enhances insulin-mediated glucose transport in vitro (Maestro *et al.*, 2000). In addition, insulin secretion is a calcium-dependent process and vitamin D metabolites have been linked to the regulation β-cell calcium pools, which promotes insulin release (Nagpal *et al.*, 2005). The putative beneficial effects of vitamin D metabolites on EF may also be explained by the mechanistic interconnection between the insulin and NO pathways. The activation of the insulin receptor on the endothelial cells induces a vasodilatory response via the activation of the phosphoinositol-3-phosphate-Akt pathway which increases NO production by the enzyme endothelial nitric oxide synthase (Siervo *et al.*, 2011a).

The meta-regression analysis showed a significant improvement of EF in response to vitamin D supplementation in participants with high BMI. There is a growing evidence of an inverse association between plasma 25(OH)D concentrations and BMI (Wortsman *et al.*, 2000; Rodríguez-Rodríguez *et al.*, 2009). Decreased vitamin D status was found in obese subjects (Wortsman *et al.*, 2000; Vilarrasa *et al.*, 2007; Rodríguez-Rodríguez *et al.*, 2009), which may be explained by adipose tissue sequestration and/ or volumetric dilution of 25(OH)D (Blum *et al.*, 2008). In turn, this may explain the tendency towards a greater effect of supplemental vitamin D on EF in subjects with greater adiposity. In addition, obesity and excess visceral adiposity are closely associated with insulin resistance and development of type 2 diabetes, which may explain the significant effect of vitamin D supplementation on EF in obese subjects. These findings support the idea that the effect size of vitamin D on EF may be correlated with the degree of metabolic derangement of the insulin signalling pathway.

Results may have been affected by the choice of the method used to measure vitamin D status i.e. by estimation 25(OH)D concentrations. Unlike chromatographic methods, immunoassays do not measure vitamin D_3 and vitamin D_2 independently, and this is a well-recognised limitation of immunoassays. The importance of being able to quantify both metabolites of vitamin D independently is becoming increasingly important with the evidence that vitamin D_3 is more biologically active than vitamin D_2 (Tripkovic et al., 2012) as well as emerging evidence that 25(OH)D_2 concentrations are in the range of 1.5-10.0nmol/L in several RCT and population-based studies and thus, may contribute significantly to total 25(OH)D status (Cashman et al., 2014). It is also important to point out that results of 25(OH)D estimation using chromatographic methods show significant variation, mainly due to extraction and calibration problems associated with these methods. Such assay variation reinforces the need for all users of vitamin D assays to have appropriate quality control and standardisation protocols in place.

This meta-analysis has some limitations. First, the available trials had relatively small sample sizes with samples sizes of <100 in about 75% of the trials included in the meta-analysis. Second, the variability in duration, dose and type of vitamin D supplementation, the different methods used to assess EF and the diversity in participant characteristics (age, sex and health status) may have introduced significant heterogeneity and have militated against observation of the true overall effects of vitamin D supplementation on EF in our meta-analysis. Third, not all studies adjusted for potential confounding factors that may have influenced the effect of

vitamin D on EF such as sun exposure, seasonality, physical activity or dietary patterns. Most of the study participants were aged between 40 to 77 years old, thus limiting the applicability of the findings to other life stages. Finally, studies have used different assays to measure 25(OH)D concentrations (Immuno-Assay, N=16; Liquid Chromatography Mass Spectrometry, N=3), which may have introduced a measurement bias **(Appendix 2.3)**. However, the exclusion of the three studies using LC-MS from the analysis did not modify the results, which provides support to the importance of vitamin D status in influencing the efficacy of vitamin D supplementation on vascular outcomes.

The current evidence base is believed to be inadequate to draw firm conclusions about any protective role of supplemental vitamin D on EF and as a pharmaco-nutritional strategy for CVD prevention. However, this study provides important information on the effects of vitamin D supplementation on EF and shows that benefit may be anticipated in diabetics and those with higher BMI. This may indicate a potential role of insulin resistance in modulating the effects of vitamin D on vascular function and a greater effect of supplemental vitamin D on EF in subjects with greater adiposity. This hypothesis remains to be tested in future studies.

Chapter 3: Methodology

3.1 Measurement of nitric oxide (NO) production

Reduced NO availability, through reduction of NO generation or NO inactivation, is an important hallmark of endothelial dysfunction, which is closely linked to the development of atherosclerosis and is an independent risk factor for cardiovascular events (van Eijk *et al.*, 2007). As a potent endogenous vasodilator, NO plays a crucial role in maintaining the homeostasis of the endothelium. However, because of the short life span of NO ($t_{½}$ = 3-6 s), the assessment of NO production can be a challenging analytical problem in humans (Kleinbongard *et al.*, 2006). This is due to its rapid chemical reaction with a wide range of molecules, e.g. NO disappears rapidly after it binds with oxyheme proteins such as oxyhaemoglobin (Hakim *et al.*, 1996) and it is also oxidized to nitrite and nitrate which have circulating half-lives of 110 seconds and 5-8 hours respectively (Bryan and Grisham, 2007).

Due to the extremely short physiological half-life of NO, the quantification of NO metabolites (nitrate (NO_3^-) and nitrite (NO_2^-)) have been used as surrogates to calculate *in vivo* NO production i.e., circulating NO_2^- can be used for the estimation of endothelial NO formation, and NO_3^- was used in estimating overall NO turnover (Kelm, 1999). However, NO can be determined using several sophisticated assays and techniques which are is not suitable for the *in vivo* setting, and are not available/ applicable for routine clinical use (Bryan and Grisham, 2007).

The methods of measurement NO production can be classified as direct methods (the target of measuring *in vivo* NO concentration itself) and indirect methods. Direct methods include the use of chemiluminescence methods to measure the real estimation of NO directly in the blood stream or in exhaled air (Siervo *et al.*, 2011c). For indirect methods, the concepts of measurements are more to the estimation of NO production rate (Mair and Puschendorf, 1998; Kleinbongard *et al.*, 2006). This involves the measurement of the concentration of NO-derived nitrite and nitrate in the blood/plasma or in urine. Indirect methods are then further classified as isotopic (van Eijk *et al.*, 2007) and non-isotopic methods, depending on whether the protocol include stable isotope tracer or not. Non-isotopic methods are then further classified into laboratory and clinical methods. Laboratory methods estimate the availability of NO by measuring the concentrations of specific substance in plasma/urine (Ahren *et al.*, 1999; Cui *et al.*, 2007). Clinical methods estimate the production of NO by measuring the vasodilatory

response of an artery which is assumed to be proportional to NO availability (Wilkinson *et al.*, 2002). The methods of measurement NO production are summarized in **Figure 3.1**. The technical characteristics, advantages and disadvantages of each method are described in **Table 3.1**.

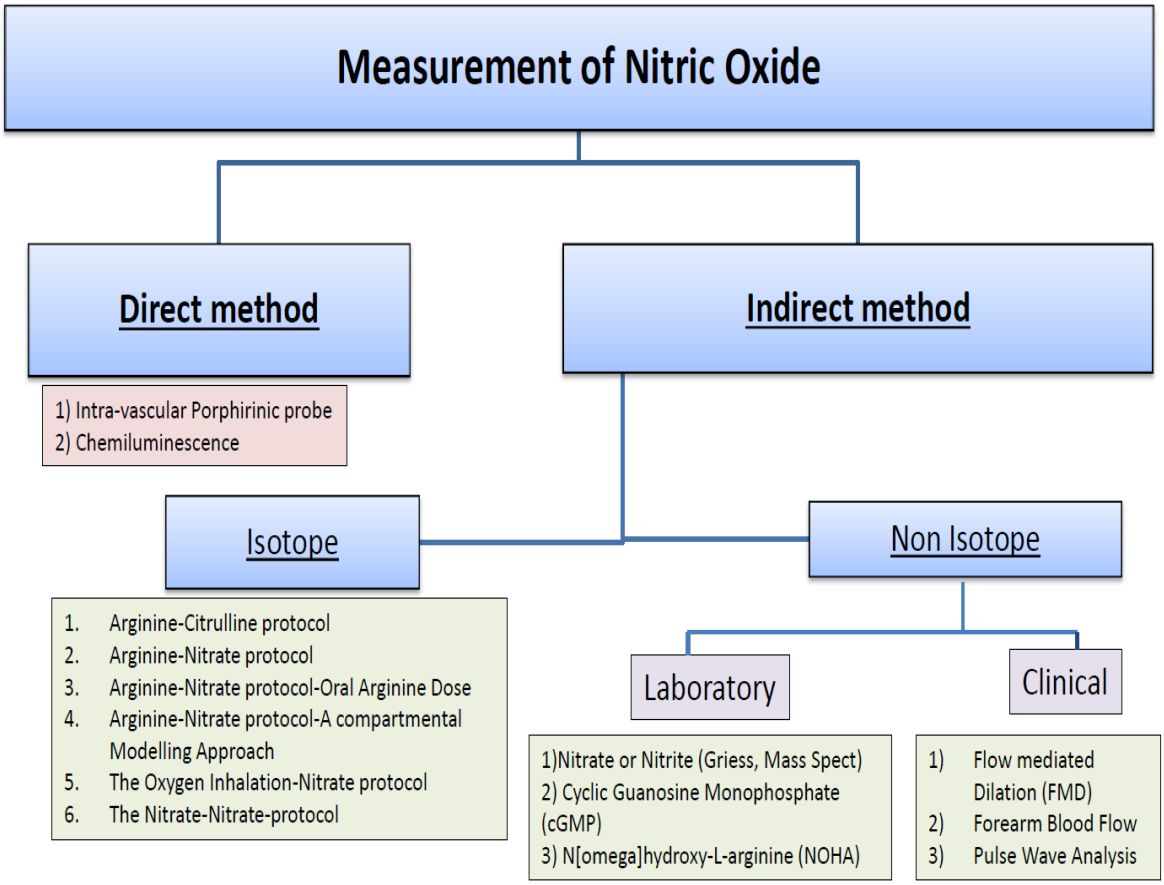


Figure 3. 1: The methods of measurement of *in vivo* NO production in humans

Table 3. 1: Description of the non-isotopic protocols used for the determination of in vivo NO production in human

Method	Description	Advantages	Disadvantages
1.Direct method			
1) Intra-vascular Porphirinic Probe (Vallance et al., 1995)	This method was invented in 1995. However, it has not been re-used in other studies for the measurement of NO. The procedure involved the insertion of a porphyrinic microsensor into a hand vein, where the vessel was then stimulated with pharmacological stimuli (acetylcholine or bradykinin).	Able to measure *in vivo* NO production in the blood vessels	Invasive and requires a high skilled operator. The estimation of *in vivo* NO production depends on the location of the infusion of acetylcholine or bradykinin and the results are site-specific, and do not apply necessarily to other parts of the vasculature.
2) Chemiluminescence (Kharitonov et al., 1997)	The measurement of *in vivo* NO concentration in exhaled breath. The principle of this method is based on the measurement of light generated by the gas-phase titration of NO and the amount light produced by this procedure is equal to the NO concentration in the air sample.	Non-invasive and convenient for use in vulnerable groups such as children and patients. Repeatable procedures	It does not give the whole estimation of NO concentration and require calibration curves to acquire the quantitative measurement. The estimation of *in vivo* NO production is dependent on the site of the measurement (respiratory system) and thus does not reflect necessarily the whole systemic NO production.

Method	Description	Advantages	Disadvantages
2. <u>Indirect method</u> **<u>Laboratory methods</u>**			
1. Nitrate or Nitrite (Griess, Ozone-based chemiluminescence, Mass Spectrometry) (Metzger et al., 2006; Tsikas, 2007)	Nitrate and NO_2^- are the relatively stable metabolites of NO found in the circulation. The concentrations of NO_3^- and NO_2^- in plasma and urinary NO_3^- can be determined using colorimetric assays based on the Griess reaction, ozone-based chemiluminescence and Mass Spectrometry.	Minimally invasive. The Griess assay is commercially available and 'ready to use' kit, which is convenient for the measurement of NO_2^-/NO_3^-. Mass spectrometry and chemiluminescence are characterised by high accuracy.	It does not provide an estimate of the rate of NO synthesis. Measurements can be influenced by confounding factors such as dietary NO_3^- intake and renal NO_3^- clearance.
2. Cyclic Guanosine Monophosphate (cGMP)(Mair and Puschendorf, 1998; Rybalkin et al., 2003)	Plasma or urine cyclic Guanosine Monophosphate (cGMP) may act as a marker of NO production. This is because cGMP is involved in the stimulation of cell proliferation and increases permeability of the vascular endothelium, inhibits cell proliferation and mediates vasorelaxation which leads to vasodilation and increased blood flow (Smolenski *et al.*, 2000; Kook *et al.*, 2003).	Minimally invasive cGMP may be quantified by ELISA which is characterised by high accuracy.	cGMP is a proxy for NO and not a direct measurement of NO concentration.
3. N[omega] hydroxyl-L-arginine (NOHA)	The principle of this method is that the compound N(omega)-hydroxy-L-arginine (NOHA) is a product of the reaction between arginine and citrulline which leads to NO production.	Minimally invasive. This metabolite is measured using high-pressure liquid chromatography (HPLC) which is characterised by high accuracy	It does not provide an estimate of the rate of NO synthesis.

Method	Description	Advantages	Disadvantages
Clinical methods ***4. Flow Mediated Dilation (FMD) (Corretti et al., 2002; Harris et al., 2010)***	FMD test characterised by the occurrence of vasodilation after the acute increase in the blood flow (shear stress resulted from the cuff occlusion in the upper arm). This leads to increases in diameter of the arterial vessel that are proportional to NO release.	Non-invasive measurement of endothelial function Repeatable measurements	Expensive equipment and highly skilled operators are required to perform the test. Confounding factors (temperature, food, drugs and sympathetic stimuli) might affect the measurement. The accuracy of the measurement is highly dependent on the quality of ultrasound images.
5. Forearm blood flow (FBF) (Wilkinson and Webb, 2001)	Also known as venous occlusion plethysmography. The principle of this measurement is that when the outflow of blood via the venous system is blocked, arterial inflow is unaltered i.e. blood can enter the forearm but cannot escape.	Accurate, reproducible and convenient method Minimally invasive	Requires expensive equipment and highly skilled operators. Does not provide a quantitative measure of NO concentration.
6. Pulse Wave Analysis (Wilkinson et al., 2002)	The principle of this measurement involves the assumption that arterial stiffness depends on the smooth muscle tone. Provides the estimation of augmentation index (AIx) measurement which can be used to assess vascular stiffness and cardiovascular risk.	Simple, repeatable, non-invasive means of assessing endothelial function in vivo. Accurate and convenient method.	The measurement of NO bioactivity is local as measurements are performed in the peripheral arterial circulation. Does not provide a quantitative measure of NO concentration.

3.1.1 Isotopic methods

Stable isotope tracers have been used for 20 years for the determination of *in-vivo* whole body NO production. Such approaches, are considered as indirect because they use precursor-end-product paradigms to characterize the pharmacokinetics of the metabolic pathway (Siervo *et al.*, 2011c). The most common protocol used in investigating NO production are summarized briefly in **Table 3.2**. Depending on the type of the protocols, estimates of the rate of NO production may differ due to differences in the route of administration, dose and rate of infusion of the tracer and the duration of the infusion protocol.

Detailed descriptions of individual protocols are beyond the scope of this PhD program, which principally focused on vitamin D, NO and vascular health. A more detailed description of the Nitrate-Nitrate Protocol is provided in Chapter 5 where it was used in the third phase of this PhD project, which explored the physiological connection between ageing, vitamin D metabolism and whole-body NO production.

Table 3. 2: Description of the isotopic protocols used for the determination of in vivo NO production in human

	Method	Description	Advantages	Disadvantages
1.	Arginine-Citrulline Protocol (Leaf *et al.*, 1989)	The principle of this measurement involved the assumption that L-arginine was the precursor for endogenously synthesized nitrate in humans.	- Collection of total urine volume is not required in this protocol. - Confounding factors from NO_3^- dietary intake can be omitted in this protocol. -The procedures in this protocol can estimate information on NO synthesis.	- Requires expensive equipment and highly skilled operators - The measurement of NO production is general and not organ-specific measures. - Invasive procedure involved the intravenous infusion ranged from 3 to 12 hours.
2.	Arginine-Nitrate Protocol (Castillo *et al.*, 1996)	This protocol uses an intravenous tracer administration (labelled arginine) as a bolus or constant infusion. Urinary NO_3^- acted as the parameter that have to be measured for the solution of the model.	- The information on NO synthesis and fractional rate of conversion of arginine into NO can be obtained from this protocol. - The assessment of NO production is less analytical challenging compared to Arginine-Citrulline protocol	- Requires total urine output collection (between 12 and 48 hours) - Restriction of dietary NO_3^- intake during the study. - Requires expensive equipment and highly skilled operators.
3.	Arginine-Nitrate Protocol - Oral Arginine Dose (Forte *et al.*, 2006)	This protocol is suitable to use in paediatric population as it is less invasive. It is based on the principles that oral dose of labelled arginine can be absorbed immediately in the GI tract and mixes with endogenous arginine pool.	- Less invasive procedure. -The information on NO synthesis and fractional rate of conversion of arginine into NO can be obtained from this protocol.	- Required collection of total urine output (36 hours). - Restriction of dietary NO_3^- intake during the study. - Requires expensive equipment and highly skilled operators.
4.	Arginine-Nitrate Protocol - A Compartmental Modelling Approach (Avogaro *et al.*, 2003)	This protocol is based on the principle of the two-compartment model can have inferences on the pharmacokinetics on the arginine-NO-nitrate pathway	- It provides estimates of the production of NO from arginine, NO production from other sources than arginine.	- Required frequent blood sampling (18 blood samples) and collection of total urine output for 48 hours.

	Method	Description	Advantages	Disadvantages
				- Requires expensive equipment and highly skilled operators. - Requires the reduction of dietaryNO_3^- intake during the study
5.	The Oxygen Inhalation-Nitrate Protocol (Sakinis *et al.*, 1999)	This protocol is based on the principle that in order to synthesis NO, oxygen is required as it will be incorporated into the NO molecule. In this protocol, subject is required to breathe a mixture of air contained in an air tight system for 30 minutes.	- Less time consuming (~30 minutes). - It provides estimates of the production of NO rate.	- Requires expensive equipment and highly skilled operators. - Requires the reduction of dietaryNO_3^- intake during the study -Blood samples are needed.
6.	The Nitrate-Nitrate Protocol (Šantak *et al.*, 1997)	This protocol has been tested in pig and demonstrated the first application of an isotopic dilution approach for the measurement of NO production	- The information of NO synthesis can be obtained. - The protocol is less analytically challenging compared to the arginine-citrulline method	- Requires the restriction of dietary NO_3^- intake during the study. - Requires expensive equipment and highly skilled operators. - Long duration procedure (~9 hours).

3.2 Assessment of endothelial function

Many methodological approaches have been developed to assess the physiological function of endothelial function in human. The assessment of endothelial function can be classified as invasive and noninvasive method (Flammer *et al*., 2010). Invasive method mainly involved the insertion of pharmacological stimuli to the forearm circulation. Noninvasive method is mainly involved the occlusion of venous drainage from the arm by placing a pressure cuff around the upper arm. The basic principle of the methods to assess vascular function (regardless invasive or non-invasive) is that healthy arteries dilate in response to pharmacological stimuli (e.g. acetylcholine, bradykinin, or serotonin) via release of NO and/or other endothelium-derived vasoactive substances, or in response to the reactive hyperemia (Flammer *et al.*, 2012). The details about each of the methods are described below. The advantages and disadvantages of the methods are summarized briefly in **Table 3.3**.

Table 3. 3: Methods, advantages and disadvantages of various assessment of endothelial function

	Method	Description	Advantages	Disadvantages
1.	*Invasive methods*			
	i.Intra-coronary artery infusion of vasoactive agents	This method involves the infusion of acetylcholine into the coronary artery. However, acetylcholine has dual effects on coronary artery tone depending on the condition of endothelium and the presence of coronary atheroma. In intact endothelium, the infusion of acetylcholine dilates normal arteries, through receptor mediated stimulation of NO production by endothelial cells (Tousoulis *et al.*, 1998). However, in endothelial dysfunction, acetylcholine decreases the vasodilatory response or vasoconstriction of the coronary artery due to the absence of NO release. This is because of its direct action on the smooth muscle cells where the constrictor effects of smooth muscle cells are dominant compared with the endothelial vasodilator effects.	The procedures in this protocol can estimate information on NO synthesis.	This method has the disadvantage as it cannot be used as a screening test in the general population due to its invasiveness and relative expense.
	ii. Intra-branchial infusion of vasoactive agents	The principle of this method is similar to the intra-coronary infusion of vasoactive agents. It involves the infusion of vasoactive substances (acetylcholine and nitroprusside) into the brachial arteries. A cannula is inserted to the branchial artery and the blood pressure and heart rate of the patients are monitored continuously. Forearm blood flow (FBF) is measured using gauge-strain plethysmography. The infusion of acetylcholine is used to evaluate the vasodilatory effect in the endothelium and changes in FBF during acetylcholine infusion reflect endothelium dilation (Virdis *et al.*, 2001).	Reliable, reproducible and easily to apply.	It has potential dangers and complications as it involves arterial cannulation (Tousoulis et al., 2005).

	Method	Description	Advantages	Disadvantages
2.	*Non-invasive methods*			
	i.Gauge-strain plethysmography	This technique evaluates changes in FBF during reactive hyperaemia. The principle of this method is the occlusion of venous drainage from the arm by placing a pressure cuff around the upper arm. The FBF output signal is transmitted to a computer. EF is evaluated after release of the pressure cuff by measuring maximum blood flow - in the artery and the percentage change from the baseline to the maximum flow. This results in a linear increase in forearm volume in the area under the curve, which represents the overall dilatory capacity of resistance arteries (Virdis *et al.*, 2001).	This method is simple, repeatable and the results are less observer dependent when compared with ultrasound-based approaches (Tousoulis *et al.*, 2005).	Requires highly skilled operators.
	ii.Flow Mediated Dilation (FMD)	This method is based on the increased of the blood flow in peripheral as the result of the changes of the diameter in the artery, after being obstructed for a certain period. This condition increased shear stress, which can reflect the ability of vascular endothelium to produce NO (Kelm, 2002). The method involves the transient ischemia in the conduit artery after a cuff is placed on the forearm and inflated for five minutes (Laclaustra *et al.*, 2007). When the cuff is deflated, the increased blood flow results in shear stress which activates eNOS and results in NO production via the L-arginine pathway. The NO released acts on the smooth muscle cells, resulting in vasodilation. FMD is measured as the percentage change in branchial diameter from the baseline to the maximum increase in diameter (Al-Qaisi *et al.*, 2008). A small FMD response is interpreted as indicating a low NO availability and possibly an associated increased risk of	Advantages of FMD includes being safe, non-invasive and reproducible and the results obtained relate closely to EF in the coronary arteries.	FMD measurement varies between assessors i.e. it is observer-dependent (Anderson and Mark, 1989).

Method	Description	Advantages	Disadvantages
	vascular disease or cardiac events (Pyke and Tschakovsky, 2005).		
iii.Laser Doppler flowmetry and iontophoresis	This technique measures the increases in blood flow after either the transdermal application of acetylcholine by iontophoresis, or the release of transient arterial occlusion (reactive hyperemia).	Sensitivity in detecting and quantifying relative changes in skin blood flow in response to a given stimulus (Kubli *et al.*, 2000).	Requires expensive equipment and highly skilled operators.
iv.Pulse wave velocity (PWV)	PWV is an indicator of arterial stiffness (Al-Qaisi *et al.*, 2008). Arterial PWV, especially in the aorta, has emerged as an important independent predictor of vascular damage and cardiovascular events (Oliver and Webb, 2003). Arterial pulse waves can be detected using pressure-sensitive transducers (Asmar *et al.*, 1995), doppler ultrasound (the pressure pulse and the flow pulse propagate at the same velocity) (Sutton-Tyrrell *et al.*, 2001), or applanation tonometry (Wilkinson *et al.*, 1998).	Simple and non-invasive, the major advantage of this technique is that it can be used to screen for vascular damage in large populations.	Does not provide a quantitative measure of NO concentration.

3.3 Measurement of biomarkers of endothelial function in biological samples

Endothelium-derived NO is an important molecule in maintaining the integrity of EF. Insufficient NO production will lead to endothelial dysfunction, which precedes cardiovascular events (Tousoulis *et al.*, 2012). In addition to measurement of NO production for assessing EF, biomarkers of endothelial activation and dysfunction may be used.

Several biochemical markers have been used as indicators of EF: The details about each of the biomarkers are described in **Table 3.4**.

Table 3.4: Biomarkers of Endothelial Function

	Biomarkers of Endothelial Function	Description
1.	Plasma concentration of NO	Circulating concentrations of nitrites and nitrosylated proteins in part reflect endothelial generation of NO, but are difficult to measure and do not always represent endothelial NO production (Rassaf et al., 2004). Many direct and indirect methods have been used to assess the production of NO (Mair and Puschendorf, 1998; Kleinbongard et al., 2006) but in vivo NO production using stable-based techniques is arguably the best global measure that reflects the underlying EF in health and disease (Siervo et al., 2011c).
2.	Asymmetric dimethylarginine (ADMA)	ADMA is an endogenous inhibitor of nitric oxide synthase, and increased concentrations of ADMA are associated with increased cardiovascular risk and endothelial L-arginine/NO pathway dysfunction (Davis et al., 2011). Plasma ADMA concentration is increased in humans with hypercholesterolemia, atherosclerosis, hypertension, chronic renal failure, and chronic heart failure (Boger, 2004). Higher concentrations of ADMA are associated with reduced NO availability in both animal and clinical studies (Vallance and Leiper, 2004).
3.	E-selectin	It is probably the most specific marker of endothelial cell activation. Higher E-selectin concentration is associated with cardiovascular risk factors, with structural and functional measures of atherosclerotic disease, as well as with adverse cardiovascular prognosis (Ridker et al., 2004).
4.	Cyclic Guanosine monophosphate (cGMP)	cGMP is a second messenger that mediates physiological changes such as proliferation, differentiation, survival, migration and apoptosis in endothelial cells. Increased cGMP is associated with vascular smooth muscle relaxation (Rapoport and Murad, 1983; Surks, 2007).
5.	Vascular Endothelial Growth Factor (VEGF)	Also known as vascular permeability factor (VPF), VEGF is important for NO release and stimulation of endothelial cell proliferation and migration. VEGF is involved in angiogenesis and vascular permeability and plays a key role in maintaining the

Biomarkers of Endothelial Function	Description
	integrity of the endothelium (Kliche and Waltenberger, 2001; Shibuya, 2011).
6. Endothelin-1(ET-1)	Whilst NO is an important vasodilator, ET-1 is a potent vasoconstrictor. Alteration or imbalance in ET-1 production has been implicated in the development of endothelial dysfunction and CVD.

In this PhD project, several methods of measuring biomarkers of EF using immunoassay have been used and the protocols are described below.

3.4 Methods of Assessing Biomarkers of Endothelial Function

3.4.1 Asymmetric Dimethylarginine (ADMA)

Asymmetric dimethylarginine (ADMA), an analogue of L-arginine, is a naturally occurring product of metabolism found in the human circulation. ADMA is produced by the methylation of arginine. Elevated ADMA inhibits the production of NO by competing with L-arginine, which is important for the synthesis of NO. The impairment of NO production leads to endothelial dysfunction and, eventually cardiovascular events (Sibal *et al.*, 2010). The measurement of ADMA in this PhD project was done using a commercial kit (CUSABIO ELISA kit CSB-E09298h) in a 96-well format. Details of the ADMA analysis procedures can be found in **Appendix 3.1**.

Principle of assay

This assay used the technique of quantitative sandwich enzyme immunoassay. The microplate has been pre-coated with an antibody specific for ADMA, which reacted with any ADMA present after the standards and samples were pipetted into the wells. The procedures involved adding the biotin-conjugated antibody specific for ADMA, washing and adding the substrate solution to develop the colour in proportion to the amount of ADMA present. The intensity of the colour was measured using a microplate reader.

3.4.2 Nitrotyrosine (3NT)

NO is important for endothelium homeostasis but excessive concentrations of NO can cause adverse effects and tissue injury (Habib and Ali, 2011). 3NT is an indicator or marker of cell

damage, inflammation and over production of NO, which can exert cytotoxic effects (Halliwell, 1997). 3NT is a well-established marker of protein damage by oxidative stress.

Peroxynitrite ($ONOO^-$) is the formation of highly reactive oxidant species, as the result of the reaction between excess NO and superoxide radical (O_2^-) (Kaur and Halliwell, 1994). It involved in the pathology of a wide range of diseases and quantification of 3NT is one of the methods for measuring indirectly oxidative stress due to the excessive production of $ONOO^-$ (Nemirovskiy *et al.*, 2009). The measurement of 3NT in this PhD project was done using a commercial kit (ABCAM ab113848) in a 96 well format. Details of the 3NT analysis procedures can be found in **Appendix 3.2**.

Principle of assay

This assay determined the concentration of 3NT modified protein in the sample. The microplate has been pre-coated with a 3NT responsive antigen. The principle of this assay is involving the amount of soluble 3NT modified protein present in the samples competes for binding by the anti-3NT detector antibody. The degree of competition is proportional to the concentration of 3NT modified protein in the sample. Therefore, the signal in each well measured by the microplate reader has an inverse relationship to the amount of 3NT in each sample.

3.4.3 Vascular Endothelial Growth Factor (VEGF)

Vascular Endothelial Growth Factor (VEGF), as its name implies, plays an important role in the homeostasis of vascular health and cardiovascular system (Khurana *et al.*, 2005). Also known as vascular permeability factor (VPF), VEGF is a signal protein produced by endothelial cell which induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis (Neufeld *et al.*, 1999). In its vasculo-protective role, VEGF up-regulates NO synthase by enhancing eNOS expression, resulted in increased synthesis of NO (Lin and Sessa, 2006). The measurement of VEGF in this PhD project was done using a 96 well format commercial kit from R&D System™ Quantikine® ELISA. Details of the VEGF analysis procedures can be found in **Appendix 3.3**.

Principle of the assay

The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF was pre-coated onto the microplate. When the standards or samples were pipetted into the wells, any VEGF presented was bounded by the immobilized antibody.

Any unbound substances were being washed, and an enzyme-linked polyclonal antibody specific for VEGF were added to the wells. The second washing was intended to remove any unbound antibody-enzyme reagent, and a substrate solution was added to the wells and colour were developed in proportion to the amount of VEGF bound in the initial step. The colour development was stopped by adding stop solution, and the intensity of the colour was measured.

3.4.4 Endothelin-1 (ET-1)

Contrary to NO which is well known as a vasodilator, ET-1 is a potent vasoconstrictor peptide isolated originally from endothelial cells. An imbalance between vasodilator (NO) and vasoconstrictor (ET-1) effects an important factor in the development of vascular dysfunction and may lead to the onset of cardiovascular disease (Agapitov and Haynes, 2002; Bourque *et al.*, 2011). The measurement of ET-1 in this PhD project was done using a 96 well format commercial kit from Enzo Life Sciences, Inc (catalog #ADI-900-020A). Details of the ET-1 analysis procedures can be found in **Appendix 3.4**.

Principle of the assay

Samples and standards were added to wells coated with a monoclonal antibody specific for ET-1. The plate was then incubated for 30 minutes. Then, the plate was washed, leaving only bound ET-1 on the plate. A solution of HRP labelled monoclonal antibody to ET-1 was added. This binds the ET-1 captured on the plate. The plate was incubated again for 30 minutes. Then, the plate was washed to remove excess HRP labelled antibody and TMB substrate solution was added. The substrate generates a blue colour when catalysed by the HRP. Lastly, stop solution was added to stop the substrate reaction and the resulting yellow colour were read at 450 nm.

3.4.5 Cyclic Guanosine Monophosphate (cGMP)

Increased concentrations of cGMP produced by guanylate cyclase within vascular smooth muscle to allow blood vessels to relax and thus increase blood flow (Ignarro *et al.*, 1986). The measurement of urinary and plasma cGMP can be an indicator for the NO production. This is because cGMP involved in the biological signalling of NO (Francis *et al.*, 2010). The measurement of cGMP in this PhD project was done using a 96 well format commercial kit from Enzo Life Sciences, Inc (catalog #ADI-900-013). Details of the cGMP analysis procedures can be found in **Appendix 3.5**.

Principle of the assay

The kit used a polyclonal antibody to cGMP to bind, in a competitive manner, the cGMP in the standard or sample which has cGMP covalently attached to it. After a simultaneous incubation at room temperature, the excess reagents were washed away and substrate was added. After a short incubation time, the enzyme reaction stopped and the yellow colours generated were read on a microplate reader at 405nm. The intensity of the bound yellow colour was inversely proportional to the concentration of cGMP in either standards or samples.

3.5 Methods to measure nitrate concentrations in biological samples

Various techniques are used in assessing nitrate and nitrite concentrations in biological samples including capillary electrophoresis, chemiluminescence, the Griess method and Gas Chromatography Mass Spectrometry (GCMS). Choosing the most appropriate method for measurement of NO and NO metabolites in biological systems depends on the background assay principle, specificity, sensitivity, advantages, disadvantages and financials (Csonka *et al.*, 2015). In this PhD study, chemiluminescence, Griess and GCMS methods were used for measuring plasma nitrate, plasma nitrite, urinary nitrate and salivary nitrate in humans. Below are the brief descriptions of the available techniques used in measuring NO_3^- concentration.

3.5.1 Chemiluminescence

Although this method is more expensive due to its high capital cost and costs for instrument maintenance, this approach has been acknowledged to be the gold standard and the most sensitive technique available to measure NO and NO metabolites (Pinder *et al.*, 2008). In this study, the ozone-based chemiluminescence method was used to measure plasma and urine NO_3^- concentrations, and plasma NO_2^- concentrations using Sievers gas-phase chemiluminescence nitric oxide analyser (NOA 280i, Analytix, UK). The main component of the NOA system include:

1) NO chemiluminescence analyser

   This machine acted as the connector for gases (N_2 and O_2), the vacuum pump, computer, printer and analog signal from the output of the analyses.
2) Purge vessel (Inert Carrier Gas)

   This custom designed glass purge vessel has an interchangeable reagent vessel (~10 ml maximum volume) with side arm injection port and a T shaped gas purging component, with a gas-tight fit to the reagent vessel **(Figure 3.3)**.

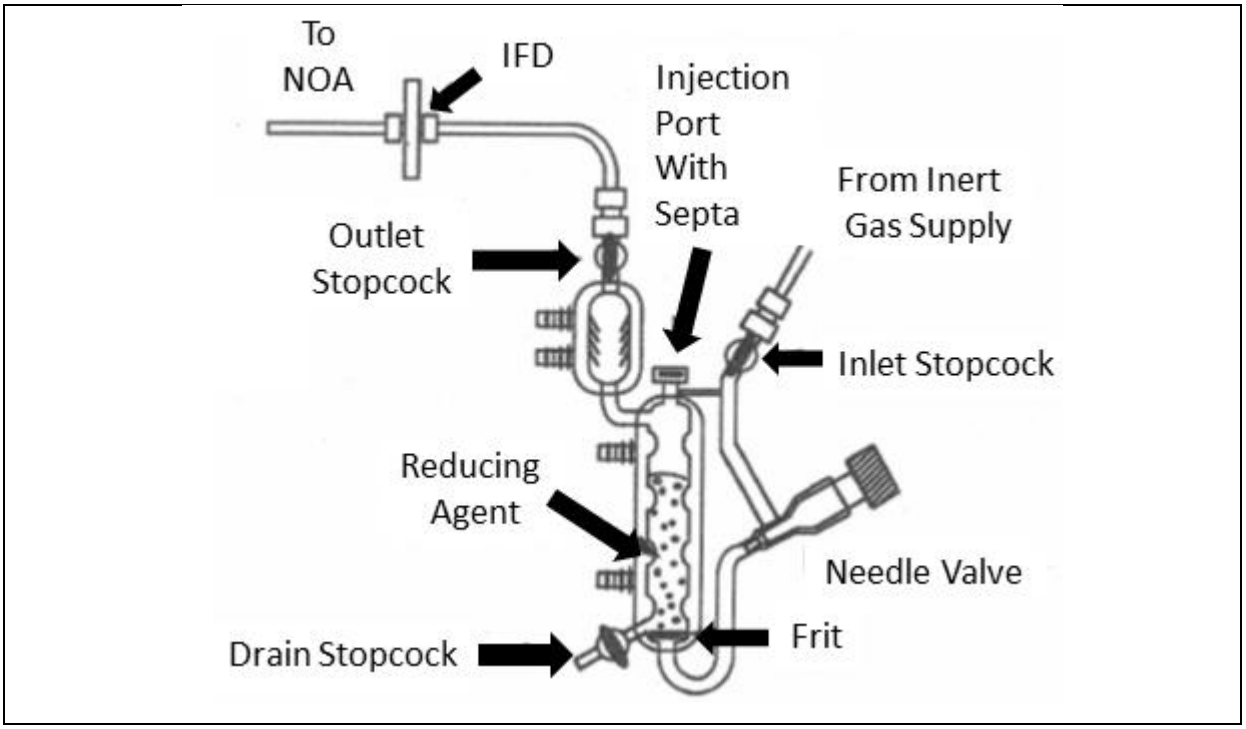


Figure 3. 2: Setup for Nitrate Measurement

Prior to the analysis, a standard curve was plotted for the calibration **(Appendix 3.6)**. The area under the curve (AUC) was measured to calculate the total NO_3^- concentrations. The Sievers® NOAnalysis™ Software Version 3.2 was used for data acquisition and data analysis.

Principle of the analysis

In the process of analysing the NO_3^- concentrations, vanadium chloride solution was added to the purge vessel. This solution reacted with the NO_3^- from the samples which were injected through the valve into the purge vessel using a glass Hamilton (Fisher) syringe. NO_3^- released from this reaction and was measured by the NOA. Details of this procedure can be found in **Appendix 3.7** and details of the NO_2^- analysis using chemiluminescence can be found in **Appendix 3.8**.

3.5.2 Griess method

Developed in 1879 by Johann Peter Griess, the Griess assay is used to measure NO and metabolites of NO (Tsikas, 2007). This is the most popular method because of its simplicity, low cost and ease of use. The assay, can be purchased in ready-to-use format and has straight forward data analysis (Hetrick and Schoenfisch, 2009; Csonka *et al.*, 2015). However, this method is more suitable for the measurement of nitrite.

Principle of assay

The analysis of nitrate requires chemical or enzymatic reduction of the nitrate to nitrite prior to the diazotization reaction. The second step is the addition of Griess reagents which convert nitrite into a deep purple-coloured azo compound. The formation of the azo compound is then monitored spectroscopically at 540 nm **(Figure 3.4)**. Details of the procedure can be found in **Appendix 3.9**.

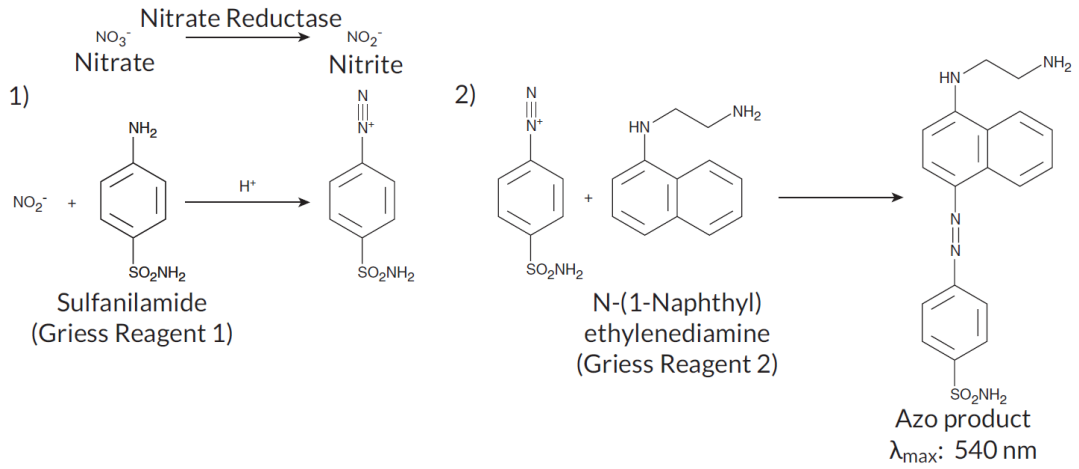


Figure 3. 3: The reaction of NO_3^- with Griess assay reagents forms an azo compound that is detected by the spectrophotometer and used to calculate the NO_3^- concentrations in the sample.

3.5.3 Gas Chromatography Mass Spectrometry

Gas Chromatography Mass Spectrometry (GCMS) has been used extensively to determine nitrate in biological samples. The first GCMS methods involved the nitration of aromatic compounds such as benzene, toluene and trimethyl-oxi-benzene (Green *et al.*, 1981; Green *et al.*, 1982; Rhodes *et al.*, 1995) in the presence of sulphuric acid, which acted as a catalyst for the reaction (Gutzki *et al.*, 1992). However, these methods, while accurate, were characterised by an important disadvantage in the utilisation of concentrated sulphuric acid which is a hazardous reagent, and could artificially increase nitrate levels due to degradation of nitrated arginine analogues (Greenberg *et al.*, 1995; Tsikas *et al.*, 1998). In this study, mesitylene (1,3,5-trimethylbenzene) was used as the aromatic electrophilic compound and trifluoroacetic anhydrate (TFAA) as the catalyst as proposed by (Jackson *et al.*, 2008). The main advantage of this approach is the combination of milder acidic conditions derived from mesitylene and

less aggressive catalyst by using TFAA which are safer and result in less interference from other compounds compared with other methods. Details on the procedure can be found in **Appendix 3.10**.

Principle of the analysis

The analysis was done using an Agilent GCMS 5977E system (Agilent Technologies, Stockport, UK) comprising a HP 6820A GC with autosampler and split/splitless injector. The GCMS was operated in Electron Ionization (EI) mode. MassHunter Data Software was used for data acquisition and data analysis. The total run time for the analysis of one sample was seven minutes. The minimum oven temperature was 90ºC and the maximum was 270ºC and the oven temperature was ramped at an increment of 30 ºC per minute to reach the maximum temperature. The peak areas were determined at m/z 165 and 166. Detailed GCMS chromatogram graph can be found in **Appendix 3.11**.

3.6 Comparison studies: Evaluation of different laboratory methods for the measurement of nitrate and nitrite concentrations in biological samples

Currently, nitrite and nitrate can be quantified in plasma, serum and urine of humans by various methods based on different analytical principles such as chemiluminescence (CL), colorimetry, spectrophotometry, fluorescence, GCMS and electrophoresis (Tsikas, 2005a). However, these methods have produced differing estimates of the concentrations of nitrite and nitrate in the circulation of healthy humans (Hunter *et al.*, 2013). One of the major reasons for these discrepancies seems to be methodological problems (Tsikas *et al.*, 1997).

This part of the chapter is dedicated to the comparison of different methods for the measurement of nitrate and nitrite in biological samples (plasma and urine). The methods used for the measurement of nitrate and nitrite are summarised in **Figure 3.5**.

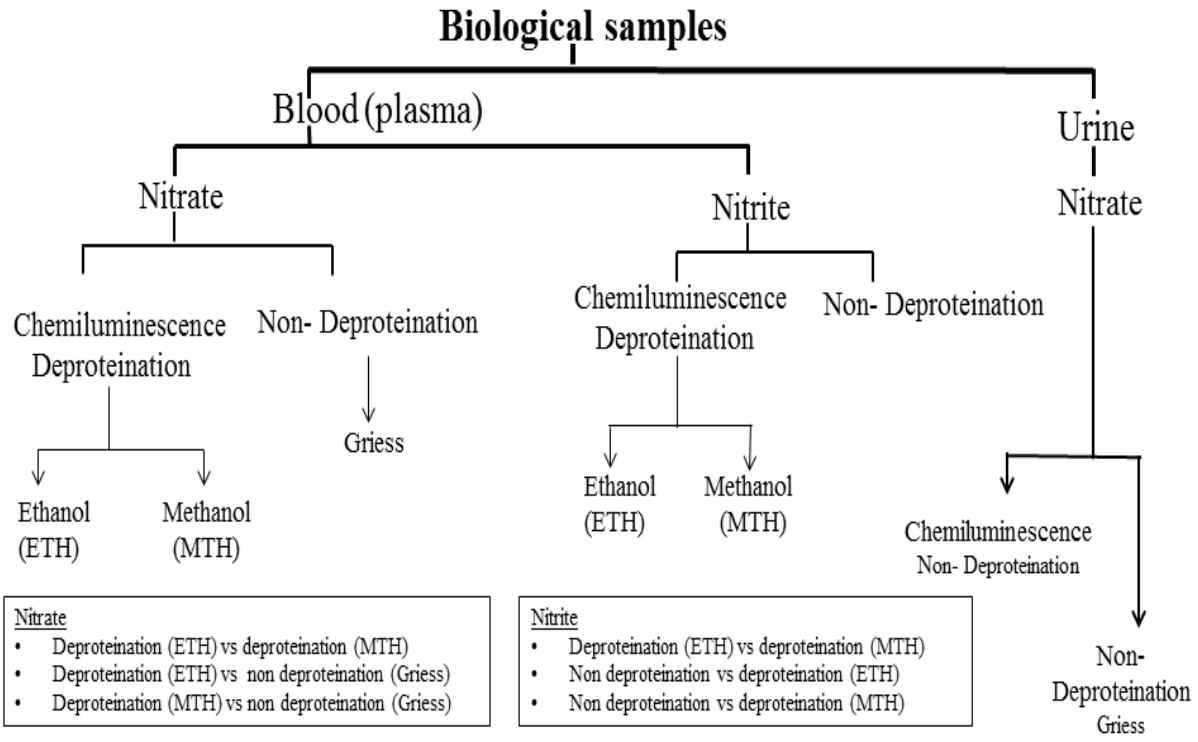


Figure 3. 4: Experiments and methods used for the measurement of nitrate and nitrite in biological samples

The following experiments were conducted to evaluate the agreement between the methods:

- Plasma nitrate (deproteination: MTH vs ETH) using CL method (n=200) (EXPERIMENT 1)
- Plasma nitrate (deproteination: MTH vs Griess) (n=80) (EXPERIMENT 2)
- Plasma nitrate (deproteination: ETH vs Griess) (n=80) (EXPERIMENT 3)
- Urinary nitrate (CL vs Griess method) (n=80) (EXPERIMENT 4)
- Plasma nitrite (deproteination: ETH vs MTH) (n=120) (EXPERIMENT 5)
- Plasma nitrite (non deproteination vs ETH) (n=120) (EXPERIMENT 6)
- Plasma nitrite (non deproteination vs MTH) using CL method (n=120) (EXPERIMENT 7)

EXPERIMENT 1: Comparison of the influence of different deproteination methods (MTH VS ETH) of plasma samples on nitrate and nitrite concentrations by ozone-based CL.

Protein precipitation, or deproteination is the process of protein removal from the plasma/serum sample prior to the analysis of nitrate/nitrite. Proteins need to be precipitated and removed from the sample otherwise these can contribute to false reading due to turbidity (Romitelli *et al.*, 2007). In addition, this procedure can reduce the foaming that occurs during the procedure due to high concentrations of protein.

For nitrate analysis in this study, deproteination methods was suggested in the chemiluminescence protocol of the GE Analytical Instrument © 2006 for Sievers NOA 280i, which are:

a) Cold ethanol precipitation (ETH)

b) Zinc Sulfate/Sodium Hydroxide precipitation (MTH)

These deproteination procedures are described below.

<u>Cold ethanol precipitation (ETH)</u>

1. The glassware and tubes were rinsed with deionized water prior the experiments to avoid any contamination.
2. The required ethanol was chilled to 0º C.
3. 0.5 mL of the sample were placed in a 1.5 mL microcentrifuge tube, 1mL of cold ethanol was added and the tube was vortexed for 10 seconds.
4. The sample was placed at 0º C for 30 minutes.
5. The sample was centrifuged at 14,000 RPM for 5 minutes.
6. The supernatant (about 1.8ml) was removed for the determination of nitrate (NB: The subsequent calculation accounted for the 3-fold dilution introduced by this deproteination method).

<u>Zinc Sulfate/Sodium Hydroxide precipitation (MTH)</u>

1. The glassware and tubes were rinsed with deionized water prior the experiments to avoid any contamination.
2. 200 µL of sample was added to the microcentrifuge tube.

3. 400 µL of 0.5 N NaOH and 400 µL of 10% by weight aqueous $ZnSO_4$ were added to the microcentrifuge tube.
4. The microcentrifuge tube was vortexed for 30 seconds and let stand at room temperature for 15 minutes.
5. The microcentrifuge tube was centrifuge at 14,000 RPM for 5 minutes.
6. The supernatant was removed for the determination of nitrate (NB: The subsequent calculation accounted for the 5-fold dilution introduced by this deproteination method).

For nitrite concentrations, the results of ETH and MTH were compared with non-deproteinised samples **(Figure 3.4)**.

1) Evaluation of the agreement between CL (reference method) and a commercially available colorimetric assay kit based on the Griess reaction (indirect method) for the measurement of nitrate in plasma and urine samples.

The samples for the plasma nitrate/plasma nitrite/urinary nitrate analyses were obtained from two studies via:

1) The cross sectional study used to evaluate the association of vitamin D with physiological and biochemical markers of endothelial function in overweight and obese post-menopausal women (described in detail in Chapter 4 of this thesis). The samples obtained from this study were used for the analysis of plasma nitrate (deproteination vs non deproteination) and urinary nitrate (80 samples).

2) An intervention study with beetroot juice supplementation which was carried out in Leeds- Beckett University - The samples obtained from this study were used for the analysis of plasma nitrate and nitrite (120 samples).

For the analysis of the effects of type of deproteination on estimates of plasma nitrate concentration, the samples from these two studies were combined to provide a total of 200 samples. Analyses of method comparison described by CL and Griess (regression equation and r^2) and by Bland Altman method (mean difference ± SD). The significance of differences between methods determined by paired *t*-test analysis is reported in **Table 3.4**.

Table 3. 4: Correlation and agreement parameters of nitrate and nitrite concentration in plasma and urine measured by the CL (deproteination and non deproteination) and Griess assay

	N	Overall mean concentration	Mean Bias	Regression equation	r^2	*t*-test (p)	+2SD	Min	Max
Nitrate-Deproteination (µM) - Chemiluminescence									
Plasma Nitrate									
Ethanol vs Methanol	200	163.08	14.11	$y = 3.4 + 0.98x$	0.83	p=0.89	93.85	-564	273
Griess vs Chemiluminescence (µM)									
Plasma Nitrate									
• Griess vs Ethanol	80	29.22	-10.92	$y = -7.11 + 0.89x$	0.77	p<0.001	10.22	-38	28
• Griess vs Methanol	80	29.18	-10.84	$y = -6.85 + 0.88x$	0.75	p<0.001	10.68	-40	37
Urinary Nitrate	80	770	-229	$y = 1.68 + 0.55x$	0.74	p<0.001	604	-4670	919
Nitrite-Non Deproteination (µM) - Chemiluminescence									
• Ethanol vs Methanol	120	602	-58.37	$y = 1.58 + 0.66x$	0.37	p<0.05	229	-891	446
• Non deproteination vs ethanol	120	427	-292	$y = -1.91 + 0.82x$	0.38	p<0.001	120	-818	72
• Non deproteination vs methanol	120	456	-350	$y = -1.04 + 0.61x$	0.37	p<0.001	218	-1154	-60

Bland Altman analysis (B & A), developed by J. Martin Bland and Douglas G. Altman in 1986 was used to assess the agreement between two quantitative methods of measurement. The principle of B & A is based on the evaluation of bias between the mean differences, and the estimation of an agreement interval. This is achieved by plotting the difference between the two methods of measurement against the average obtained with each of the two techniques (Bland and Altman, 1986). Good agreement between the two methods exists when the mean of the difference is close to zero and the individual differences are within the limits of agreements, i.e. within the range mean ± 1.96 SD. The regression line is drawn to evaluate bias between the two methods. Horizontal lines are drawn at the mean difference, and at the limits of agreement, which are defined as the mean difference plus and minus 1.96 times the standard deviation of the differences.

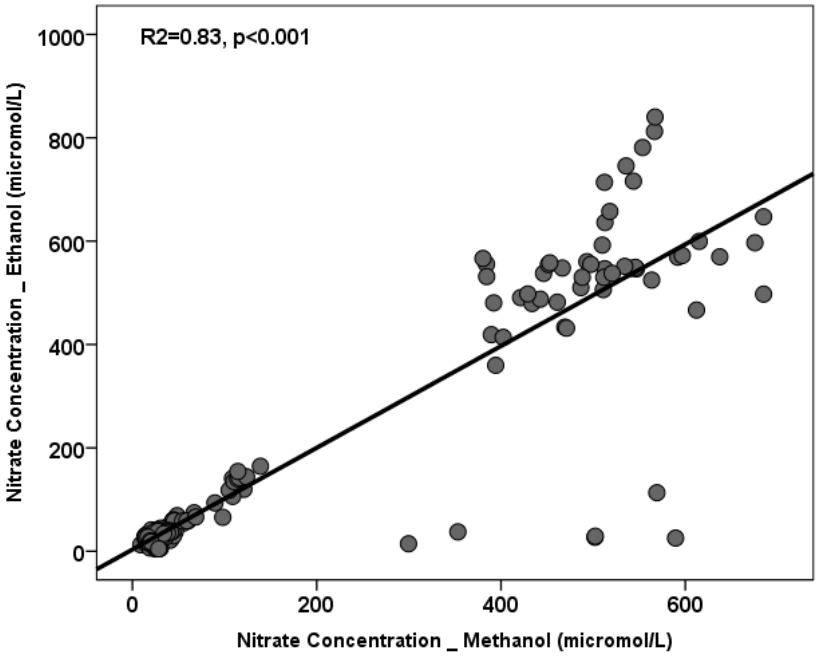
In this study, the B & A method has been used to provide a visual representation of the agreement between the methods of measuring plasma nitrate/ plasma nitrite/urinary nitrate. The analysis was conducted in SPSS 22 for Windows (SPSS, USA) and Excel for Windows (Microsoft Corporation, USA).

3.6.1 Estimation of Plasma Nitrate concentration

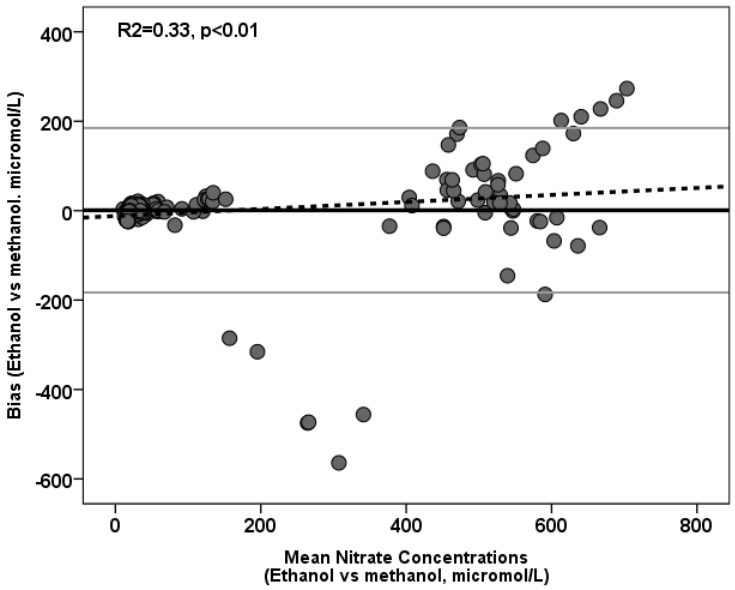
1) CL (Deproteinisation: ETH vs MTH – Experiment 1 and Experiment 2)

The results of the comparison between two methods of deproteination (ETH vs MTH) using CL are summarised in **Figure 3.6**. **Figure 3.6 (A and B)** showed a strong, significant association between plasma nitrate concentrations measured by the MTH and ETH deproteinisation methods (n=200, r=0.83, p<0.001). The BA plot showed that the majority of the data lie within the 95% CI. However, there are five clear outliers in the scatterplot and the removal of these points further improved the association between the two methods (n=196, r=0.97, p<0.001) **(Appendix 3.12)**.

The difference in estimates between MTH and ETH methods used for measurement of nitrate concentrations in plasma samples was small (mean bias = 0.84, p=0.89) **(Table 3.4)**. In addition, B & A analysis confirmed this small difference between the two methods with majority of the points lying within the limits of agreements. However, the bias in estimates between the two methods increased as the concentration of nitrate increased at the average of 200 µM (R^2=0.33, p<0.01) **Figure 3.6 (B)**.



(A) Regression analysis of plasma nitrate concentration (µM) estimated by CL after deproteination using the ETH and MTH methods



(B) Bland-Altman analysis of estimates of plasma nitrate concentration after deproteination using the ETH and MTH methods

Figure 3. 5: Comparison of CL for deproteination method using ETH and MTH.

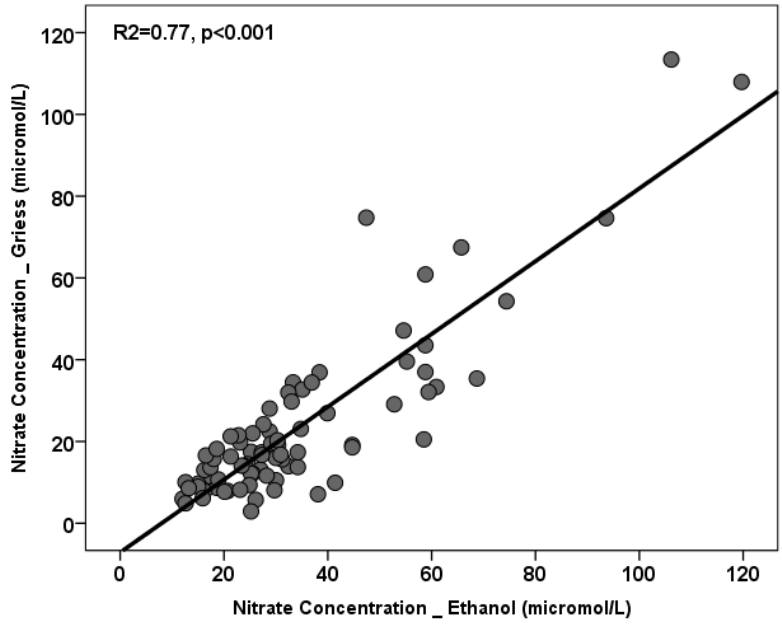
(A) Linear regression analysis between plasma nitrate concentration observed by the ETH and MTH. (B) B & A method was used to evaluate the agreement between the nitrate concentrations measured by the ETH and MTH. Black horizontal lines (bold) show the mean difference between ETH and MTH and the fine, grey line, are the limits of agreement (± 2SD). A regression line was fitted to the points (dashed black line) to evaluate differential bias.

Recommendation for practice:

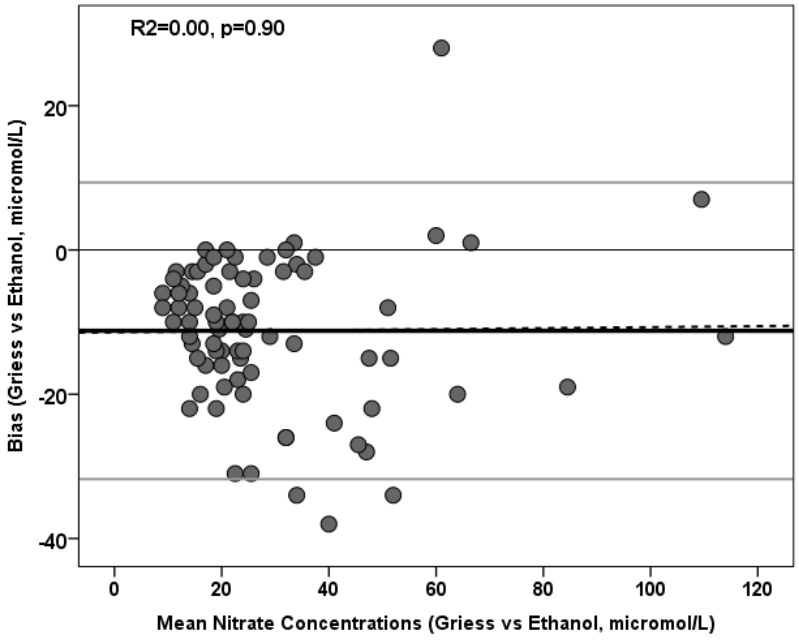
Given the negligible, non-significant difference (p=0.89) in estimates of plasma nitrate concentration after use of the two deproteination methods, it is suggested that either ETH or MTH method can be used for nitrate concentration measurement using the CL procedure. This procedure is suitable to be used for concentration of nitrate up to 200 µM.

2) CL (deproteination: ETH) vs Griess (non-deproteination)- Experiment 3

The objective of this experiment is to assess the comparison between the two methods of nitrate analysis by CL (samples were processed using the ETH method) and Griess method **(Figure 3.7 A and B).** Linear regression analysis showed a strong significant association between the two methods (n=80, R^2=0.772, p<0.001**, Figure 3.7 A**). The results from the B & A analysis showed that nitrate concentrations in plasma sample measured by the Griess method were significantly higher than the CL method (mean bias = -10.92, p <0.001) **(Table 3.4)**. The BA plot showed that the majority of the data lie within the 95% CI. Most of the points lied within the limits of agreement and we found no evidence of differential bias with increasing nitrate concentrations (R^2=0.001, p=0.90 **Figure 3.7 B**). Similar results between the two methods were seen if plasma samples, measured by CL, were processed by the MTH method (See figure – in **Appendix 3.13**).



(A) Regression analysis of plasma nitrate concentration (µM) estimated by CL and Griess



(B) Bland-Altman analysis of estimates of mean plasma nitrate concentration using CL and Griess methods

Figure 3. 6: Comparison of CL and Griess methods for the measurements of plasma concentrations in 80 subjects.

(A), Linear regression analysis to evaluate the association between plasma nitrate concentration measured by the Griess and CL methods. (B), Agreement and differential bias between the two methods was evaluated by the Bland-Altman method. Black horizontal lines (bold) show the mean difference between CL and Griess methods and the fine, grey line are the limits of agreement (± 2SD). A regression line was fitted to the points (dashed black line) to evaluate differential bias. Plasma samples measured by CL were processed using the ETH method.

Recommendation for practice:

Even though we found a strong significant association between the two methods, the Griess method over-estimated the nitrate concentrations compared to the CL approach. Therefore, results from Griess method should be interpreted cautiously.

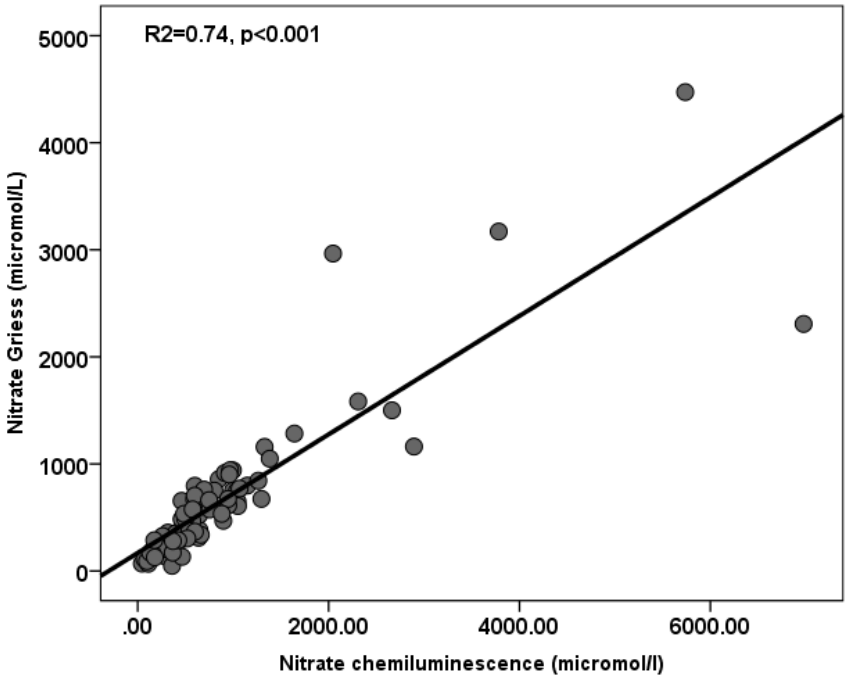
3.6.2 Estimation of Urinary Nitrate Concentration

1) CL (non depoteination) vs Griess (non deproteination) –Experiment 4

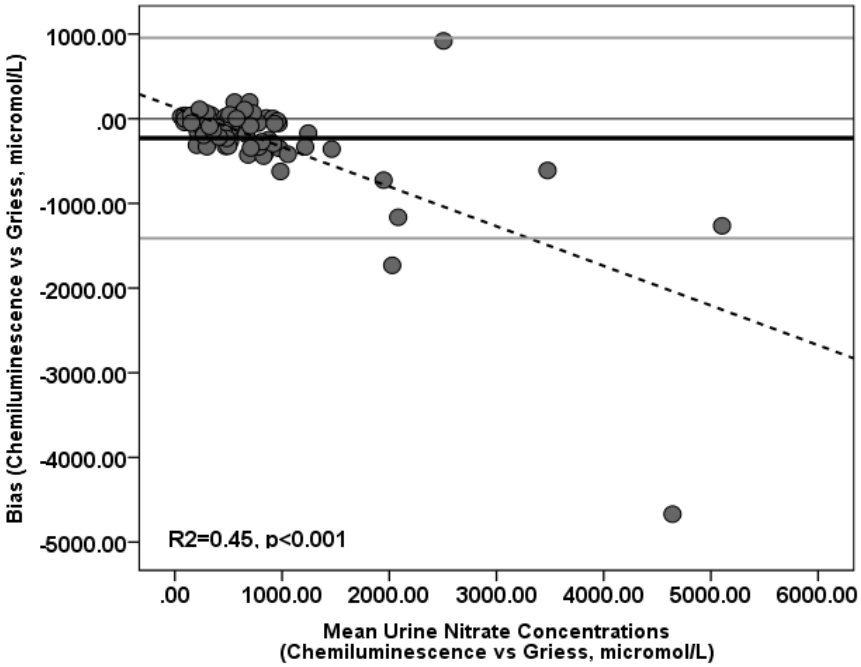
The objective of this experiment is to assess the agreement between the two methods of urinary nitrate analysis by CL and Griess method. Linear regression and B & A analyses were performed.

The results for the comparison of the two methods were illustrated in **Figure 3.8 A and B**. Linear regression analysis of urinary nitrate concentrations (in µM, range 44-6977 µM) measured by Griess (y) versus those measured by CL (x) revealed a significant correlation between the two methods (n=80, R^2= 0.74, p<0.001) **(Figure 3.8 A)**.

The B & A analysis showed a significant difference was found for the measurement of urinary nitrate concentrations as the Griess significantly overestimated nitrate concentrations compared to CL (mean bias= 228.94 nmol/L; p<0.001) **(Table 3.4)**. The BA plot showed that the majority of the data lie within the 95% CI. In addition, a significant differential bias as the difference between the two methods increased as urinary nitrate concentration increased (R^2=0.45, p<0.001) **(Figure 3.8 B)**.



(A) Regression analysis of urinary nitrate concentration (µM) estimated by CL and Griess



(B) Bland-Altman analysis of estimates of mean urinary nitrate concentration using CL and Griess methods

Figure 3. 7: Comparison of CL and Griess methods for the measurements of urinary nitrate.

(A), Linear regression analysis to evaluate association between Griess and CL methods. (B), B & A plot to assess the agreement between the Griess and CL methods for measuring the difference in nitrate concentrations. Black horizontal line shows the mean difference (bold) and the ± 2S.D. range (fine, grey line). A regression line was fitted to the points (dashed black line) to evaluate differential bias.

Recommendation for Practice:

Similar to plasma nitrate analysis, the Griess method over-estimated urinary nitrate concentration compared with the CL method. Furthermore, the difference between these two methods increased in line with the increased of urinary nitrate concentration. Therefore, it can be recommended that results from Griess method should be interpreted cautiously.

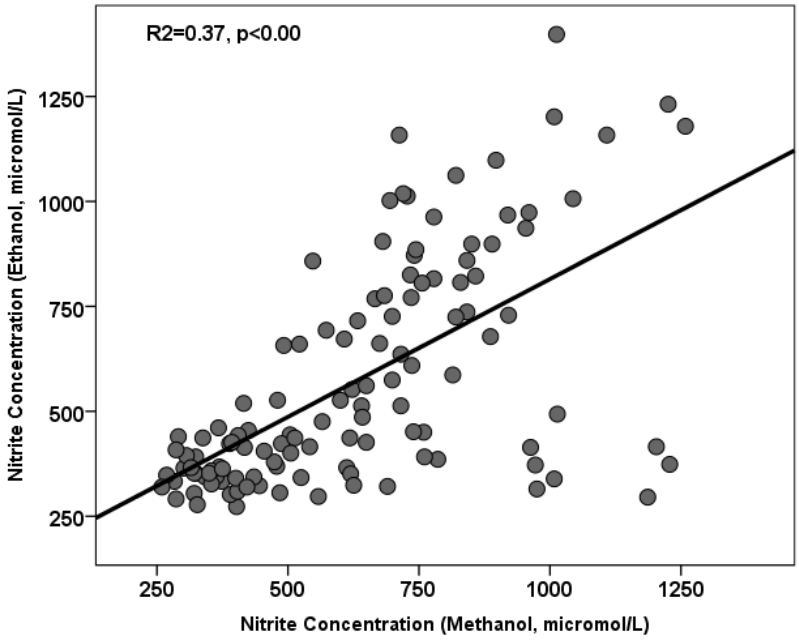
3.6.3 Estimation of Plasma Nitrite concentration

1) CL (deproteination: ETH vs MTH) – Experiment 5

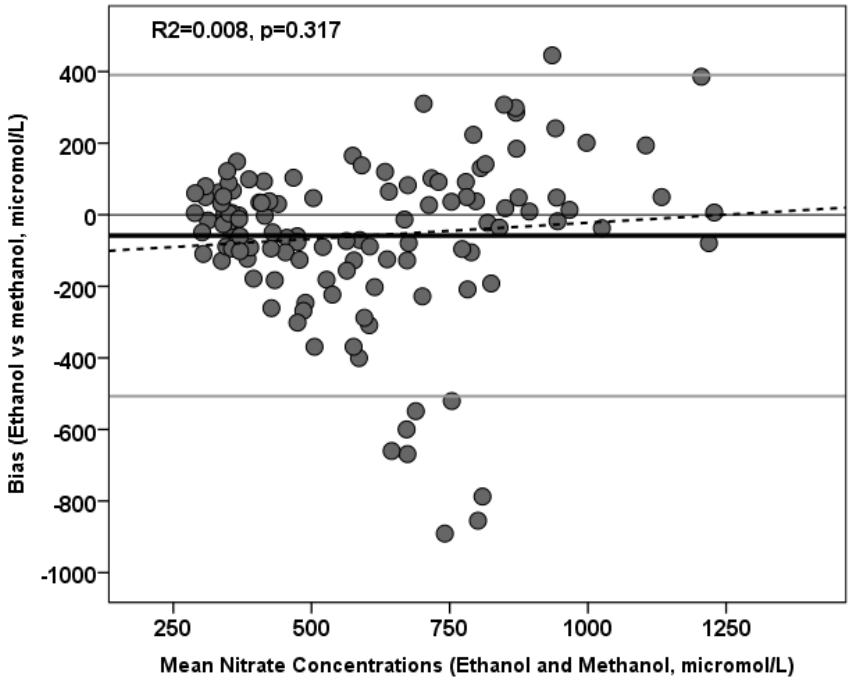
The objective of this experiment is to assess the correlation and agreement between the two methods of deproteination (ETH vs MTH) using CL. B & A method was performed to describe the agreement between the two methods.

The results for the comparison of the two methods of deproteination are illustrated in **Figure 3.9**. **Figure 3.9 (A and B)** and show the agreement in plasma nitrite concentration between the two methods. Linear regression analysis revealed a significant correlation between the two methods ($n=120$, $R^2=0.37$, $p<0.001$, **Figure 3.9 A**). However, the B & A analysis showed a significant difference for the measurement of nitrite concentrations between the MTH and ETH nitrite methods (mean bias= 58.3nmol/L; $p<0.05$) **(Table 3.4)**.

The BA plot showed that the majority of the data lie within the 95% CI. The majority of the data points were between the limits of agreements, and no evidence of differential bias has been found with increasing nitrite concentrations ($R^2=0.008$, $p=0.317$, **Figure 3.9 B)**.



(A) Regression analysis of plasma nitrite concentration (μM) estimated by CL (ETH vs MTH)



(B) Bland-Altman analysis of estimates of mean plasma nitrite concentration using CL (ETH and MTH)

Figure 3. 8: Comparison of effects of ETH and MTH deproteination methods on the measurement of plasma nitrite by CL.

(A), Linear regression analysis to evaluate associations between the two methods. (B), B & A plot to assess agreement between ETH and MTH deproteination methods for the measurement of differences in nitrite concentrations. Black horizontal line shows the mean difference (bold) and the ± 2S.D. range (fine, grey line). A regression line was fitted to the points (dashed black line) to evaluate differential bias.

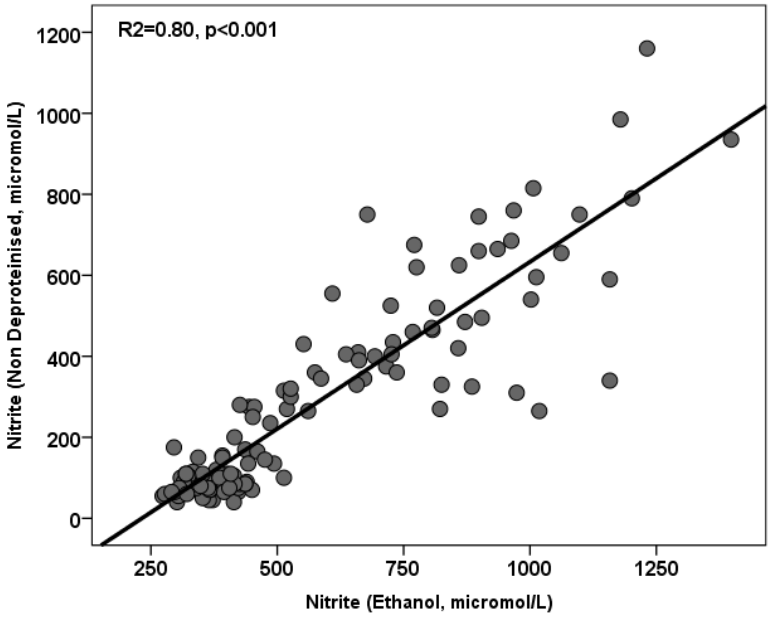
Recommendation for practice:

Referring to the significant influence of plasma nitrite between the two deproteination methods, where a slightly higher of NO_2^- concentration measured using MTH method, it is suggested that ETH method should be used for NO_2^-concentration measurement using CL procedure.

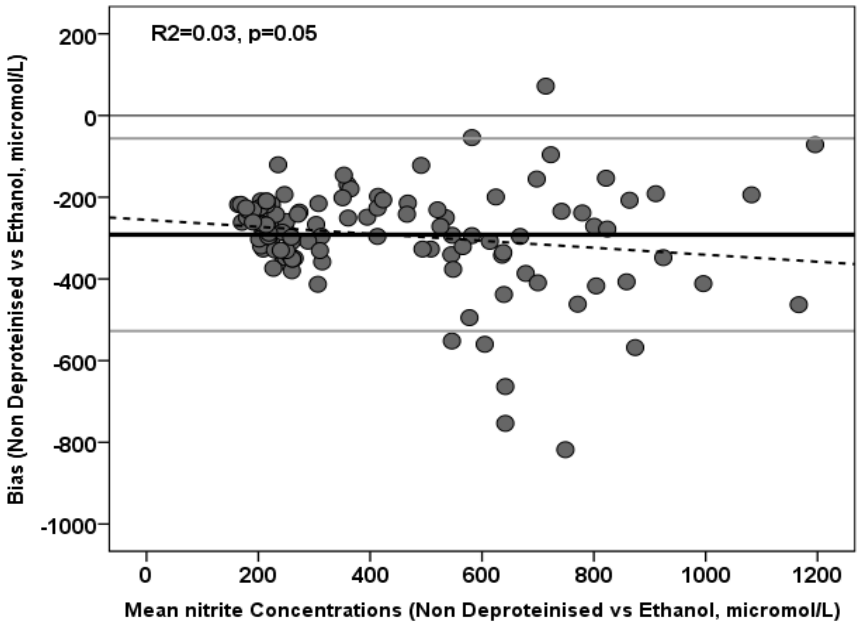
2) CL (deproteination: ETH and MTH) vs CL (non deproteination) –Experiment 6

The objective of this experiment is to assess the agreement between the two ETH deproteination methods and untreated (non-deproteination) plasma samples for the measurement of nitrite by CL. The results for the comparison of the two methods were illustrated in **Figure 3.10**.

Linear regression analysis of plasma nitrite concentrations (in µM, range 40-1398 µM) showed a significant correlation between the two methods (n=120, R^2=0.80 p<0.001, **Figure 3.10 A)**. However, there was a significant difference between the two methods and estimates of the nitrite concentrations from the non-deproteination method were significantly higher than those found using the ETH method (mean bias= 291.8 nmol/L; p<0.001) **(Table 3.4 and Figure 3.10 B)**. The BA plot showed that the majority of the data lie within the 95% CI. However, no evidence of a differential bias was found between the two methods (R^2=0.03, p=0.05) **(Figure 3.10 B)**. Similar results were obtained from the application of the MTH deproteination method **(Appendix 3.14)**.



(A) Regression analysis of plasma nitrite concentration (µM) estimated by CL (untreated vs ETH)



(B) Mean plasma nitrite by CL (untreated vs ETH) Bland-Altman analysis of estimates of mean plasma nitrite concentration using CL (untreated vs ETH)

Figure 3. 9: Comparison of plasma nitrite concentration measured by CL in untreated samples and using the ETH deproteination method.

(A), Linear regression analysis to evaluate the association between the two processing methods. (B), B & A method to assess agreement between the two methods and evaluate presence of differential bias. Black horizontal line shows the mean difference (bold) and the ± 2S.D. range (fine, grey line). A regression line was fitted to the points (dashed black line) to evaluate differential bias.

Recommendation for practice:

It is suggested that the process of deproteination is crucial in analysing plasma nitrite. This is because it will reduce foaming formation and improves applicability of techniques. Non-deproteinised samples had higher nitrite concentrations than those that were deproteinised.

3.6.4 Discussion

These experiments were dedicated to explore the comparison of different methods for the quantitative analysis of nitrate and nitrite in biological samples (plasma and urine). As been discussed earlier in this chapter, there are various analytical methods based on different analytical principles, such as colorimetry, spectrophotometry, fluorescence, chemiluminescence, gas and liquid chromatography, electrophoresis and mass spectrometry (Tsikas, 2005a). The method for the assessment of nitrate and nitrite in humans depends on the availability of resources and equipment, technical and research expertise.

In this study, CL (with and without deproteination) method and the Griess method were used for quantifying concentrations of nitrate and nitrite in plasma and urine. These methods were used based on the availability of the machine in the laboratory (ozone based CL) and the commercially available of 'ready to use' kits for the Griess method. In addition, the GCMS method was used for the analysis of saliva samples in the third phase of this PhD project (details in Chapter 5).

In general, following comparison of the three techniques (CL, Griess and GCMS) used in measuring nitrate/nitrate in plasma, urine and saliva samples, the following conclusions have been made:

1) Even though CL is the best technique for measuring nitrate (Piknova *et al.*, 2016), extra precaution is required when using this method. Injecting the samples into the purge vessel resulting in excessive bubbles even though an antifoaming agent was used, and this was exacerbated with for non-deproteinised due to the protein present in the samples. The bubbles should be monitored by adjusting the needle valve so that the bubbles did not get into the inlet tube since that will damage the machine. At maximum of 10 injections of samples were done before the nitrate reducing agent solution in the bubble base needed to be changed. The cell pressure should be constantly monitored at

4 – 7 torr. The samples need to be deproteinised prior to the analysis, which may add considerable time and expense to the analysis. Due to tedious, time consuming and technically difficult, it can be suggested that CL method is only suitable to be used in research laboratory setting.

2) The Griess method is practical for analysing large numbers of samples where the fast, cheaper and reproducible measurement is a considerable advantage. However, the presence of protein in the sample, can interfere in the outcome. Therefore, performing protein removal prior to the Griess procedure might be beneficial into reducing inaccuracy in the determination of nitrate/nitrite concentrations in samples (Hunter *et al.*, 2013).

3) GCMS, although quite cumbersome to performed, is considered the most accurate technique of measurement nitrate/nitrite in biological samples (Romitelli *et al.*, 2007). In the study reported in the Chapter 5 of this thesis, the saliva samples have to undergone several steps of derivatisation before being analysed in the GCMS machine. Drying down, heating, washing and filtration were the procedures done before the sample were placed into the injection port of the GC device. Due to the multiple derivatisation procedures prior to the measurement, time consuming and expensive equipment, it can be suggested that GCMS is more suitable to be used in the clinical and research laboratory setting and performed by experienced personnel.

Discrepancies and methodological problems on diverging values of nitrate/nitrite measured are depends on the analytical principles of the method (Tsikas, 2005b). Tsikas et al reported a relatively poor correlation (R= 0.8) between estimates of nitrate in human urine samples (n=33) when estimated using the Griess assay and by GCMS (Tsikas *et al.*, 1997). This discrepancy may be due to the interference by free reduced thiols, proteins, and other plasma constituents in the Griess assay.

There are various methods of deproteination available for treating plasma samples prior to nitrate/nitrite analysis such as *acetonitrile-chloroform*, ultrafiltration, *zinc sulfate, zinc sulfate-sodium hydroxide* and *methanol-diethyl ether*. The complete removal of protein is essential because any remaining protein may cause turbidity and may lead to the inaccuracy in determination of nitrite and nitrate concentration. In this study, authors suggested that

acetonitrile-chloroform is the most efficient method for protein precipitation (Romitelli *et al.*, 2007).

In the present PhD study, for nitrate analysis using CL method, there was no difference in the effects of MTH and ETH as deproteination agents prior to the measurement of nitrate concentrations in plasma samples **(Figure 3.6)**. However, for nitrite analysis using CL method, nitrite concentrations were significantly higher after using the MTH than the ETH approach **(Figure 3.9)**. However, both deproteination methods resulted in lower plasma nitrite concentrations than were observed when non-deproteinised samples were analysed. These results suggest that both ETH and MTH can be used as deproteination agents for nitrate measurements. Deproteination using ETH method is adviced for the measurement of nitrite concentrations as it produced less foaming and shorter protocol for the processing of the samples. While non-deproteinised samples had higher nitrite concentrations, the formation of foam makes the analyses cumbersome and technically difficult.

The comparison between the Griess method and the CL method showed relatively poor agreement for the measurement of nitrate in both plasma and urine. Both deproteination methods (ETH and MTH) showed a similar degree of under-estimation of plasma nitrate concentrations by the Griess compared with CL **(Figure 3.7)**. Finally, the Griess method significantly over-estimated urinary nitrate concentrations **(Figure 3.8)**.

For the analysis using Griess method, we can conclude this method performs poorly in plasma and urine samples for the measurement of nitrate concentrations and its results should be interpreted cautiously. In the case of proportional difference variability between measurements, i.e. constant coefficient of variation across the range of concentration, the effect on the B & A revealed that in all experiments performed, all unit difference has a widening trend of the agreement range with increasing concentrations on nitrite/nitrate in urine/plasma.

3.6.5 Conclusion

In conclusion, even though analysis of nitrate/nitrite concentrations using the Griess method is tempting due to its simplicity, low cost and ease of use, results from the CL method are more reliable because it is one of the most sensitive methods to measure low nanomolar concentrations of metabolites. This study also found that there is no significant difference between the two methods of deproteination (ETH and MTH) when used for nitrate analysis,

suggested that these two methods can be used interchangeably. However, when undertakings nitrite analysis, the ETH method is the preferred method of protein precipitation.

3.7 Comparison studies: Evaluation of accuracy of laboratory methods for the measurement vitamin D concentration using a conventional method (assays on liquid serum collected by venepuncture) and using a dry blood spot (DBS) method

In the cross sectional study entitled - "Association of vitamin D with physiological and biochemical markers of endothelial function in overweight and obese post-menopausal women" (described in detail in Chapter 4), the analysis of 25(OH)D concentration was done using two methods:

1. A conventional method using chemiluminescence immunoassay (CLIA) on liquid serum collected by venepuncture.
2. A more novel method based on analysis of dry blood spots (DBS).

Below are brief descriptions of the analyses used with these two approaches.

3.7.1 Analysing of 25(OH)D concentration using the conventional method.

In this study, serum from use of plain blood collection vacutainer tubes was used for assay of 25(OH)D using a competitive immunoassay utilizing CLIA technology at the Freeman Laboratories, Freeman Hospital, Newcastle upon Tyne NE7 7DN. Details of the procedure or analysing 25(OH)D concentration using this method can be found in **Appendix 3.15**.

3.7.2 Analysing 25(OH)D concentration using DBS

The use of DBS is a relatively new approach for collection and storage of blood samples for later measurement of a wide range of analytes including 25(OH)D concentration. The blood samples can be self-collected using a minimally invasive technique which has the advantages that it does not require a phlebotomist to perform the venepuncture, and the samples do not require centrifugation and low temperature storage, which are crucial in the traditional method to prevent the degradation of plasma or serum samples (Hoeller et al., 2016). The procedure involved using a small sterile lancet to prick the skin and to produce a drop of blood which is dropped on to a marked circle on a filter paper card (Chambers et al., 2015). The blood is allowed to dry at the room temperature and then the DBS card is stable at room temperature. This means that the DBS system can be used for blood sample collection in populations at remote locations or at home, and the shipment of frozen samples is unnecessary (Demirev,

2013). One explanation for this may be the presence of a specific vitamin D binding protein in blood that, together with serum albumin, bind >99 % of the circulating25(OH)D, thereby stabilizing the vitamin on the DBS cards (Hoeller et al. 2016).

For this study, five circles on the filter card (Whatman Protein Saver 903 card; GE Healthcare from DSM Nutritional Products Ltd. Switzerland) were required to be filled with blood. For the finger prick, 2.0-mm contact-activated lancets (BD Microtainer; becton, Dickinson and Company) were used. The DBS cards were then air dried for at least one hour but not more than four hours at room temperature before being stored in the laboratory at -80ºC. The analyses of DBS were done at City Assay, Clinical Biochemistry Sandwell and West Birmingham Hospitals NHS Trust, City Hospital, Dudley Road, Birmingham, B18 7QH. Details of the procedure used can be found in **Appendix 3.16**.

This part is dedicated to the comparison of results of 25(OH)D concentrations obtained after these two methods. For that purpose, Bland Altman method was performed to provide a visual representation of the agreement these two methods.

The samples for the 25(OH)D were obtained from two studies;

1) Study 1 - Cross sectional study to evaluate the association of vitamin D with physiological and biochemical markers of endothelial function in overweight and obese post-menopausal women (described in detail in Chapter 4 of this thesis).

2) Study 2 - The effect of vitamin D and physical activity on knee osteoarthritis symptoms in older obese adults: a mixed methods study (PhD thesis, R Brown, Newcastle University 2017 - Personal communication from JC Mathers).

The results of the 25(OH)D analyses conducted using the two methods are summarised in **Table 3.5**. In the two studies, there was a significant difference between 25(OH)D concentrations derived by direct analysis on liquid serum after venepuncture and those derived using DBS method. On average, 25(OH)D concentrations derived from DBS were more than two fold higher compared with direct analysis of serum sample in both studies.

Table 3. 5: Mean 25(OH)D concentrations measured from serum sample and DBS in two independent studies

	Mean (SD)	Min - Max	P value
Study 1 (n=80)			
Serum sample	44.84 (23.87)	12 - 125	***p*<0.001**
DBS sample	101.78 (40.02)	40.20 – 218.90	
Study 2 (n=20)			
Serum sample	67.35 (29.52)	13.00 – 122.00	***p*<0.001**
DBS sample	147.98 (54.71)	23.40 – 218.40	

Significant p values are shown in bold.

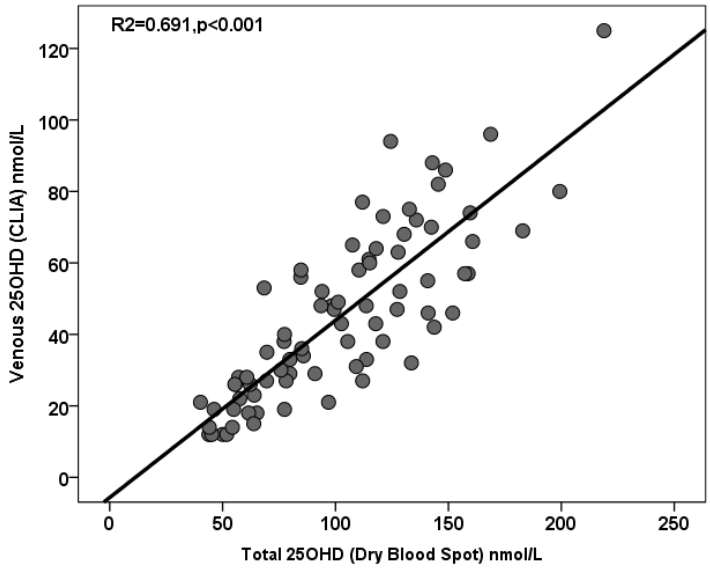
Table 3.6 summaries the correlation and agreement of 25(OH)D concentration obtained using the two methods.

Table 3. 6: Correlation and agreement parameters of 25(OH)D concentration in serum measured by conventional and dry blood spot method

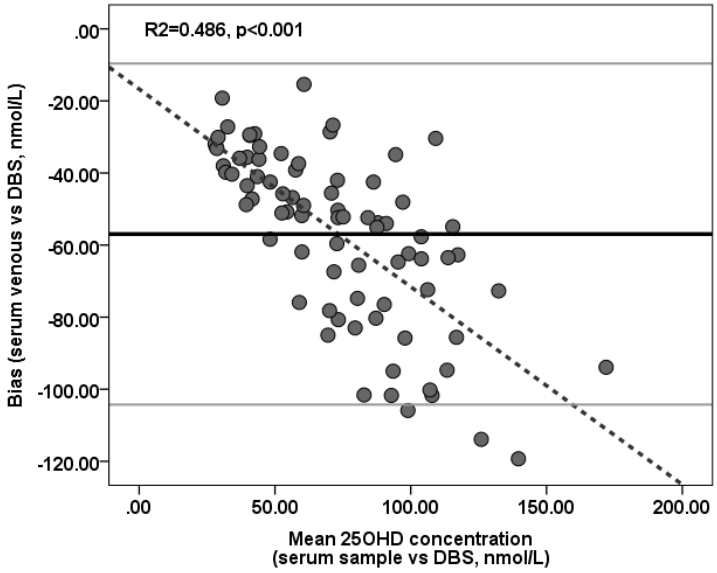
	N	Mean Bias	Regression equation	r^2	*t*-test (p)	+2SD	Min	Max
Study 1	80	-56.94	y=-5.65+ 0.5x	0.691	p <0.001	24.14	-119.3	-15.40
Study 2	20	80.63	y=9.96 + 0.39x	0.47	p <0.001	39.28	-41.60	137.8

Study 1 (n=80)

The results of B&A analysis for the comparison of the two methods are illustrated in **Figure 3.11.** This showed that, whilst there was a strong and significant, positive correlation between 25(OH)D concentrations measured by the conventional and DBS methods (n=80, r^2=0.69, p<0.001), the DBS approached significantly and systematically overestimated 25(OH)D concentrations compared to the conventional method (mean bias= 56.94 nmol/L; p<0.001) **(Table 3.6)**. The 95% limits of agreement are -9.62 nmol/L, -104.26 nmol/L and all of the points are within these limits apart except for 3 points. For these three points, which is outside the 95% limit, the 25(OH)D concentrations of DBS are much higher than that given by venous 25(OH)D. In addition, a significant differential bias was found as the difference between the two methods increased with a rise in 25(OH)D concentrations (R^2=0.486, p= <0.001) **Figure 3.11 B.**



A) Regression analysis of 25(OH)D concentration (nmol/L) by conventional and DBS method



B) Bland-Altman analysis of estimates of mean 25(OH)D concentration (nmol/L) serum venous vs DBS

Figure 3. 10: Comparison of 25(OH)D concentration using serum venous (conventional method) and DBS method.

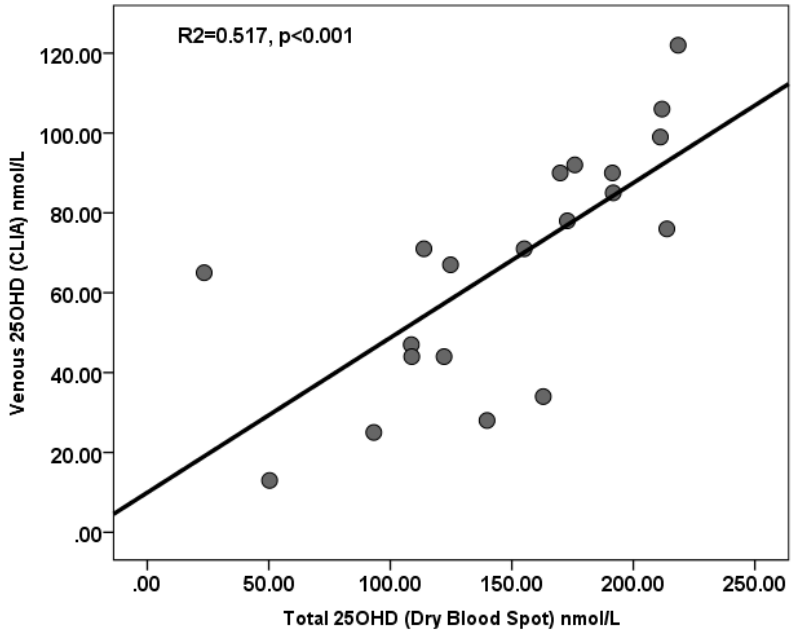
(A), linear regression analysis between venous 25(OH)D and DBS (B), Agreement and differential bias between the two methods was evaluated by the Bland Altman method. Black horizontal lines (bold) show the mean difference between conventional and DBS method and the fine, grey line are the limits of agreement (± 2SD). A regression line was fitted to the points (dashed black line) to evaluate differential bias.

Recommendation for practice:

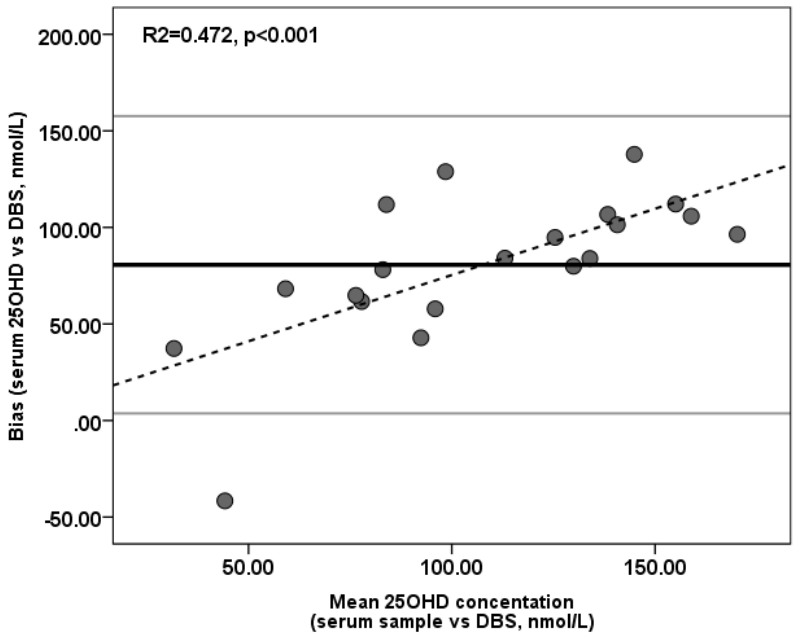
In this study, the DBS method over-estimating 25(OH)D measurement compared with the conventional method. Furthermore, the difference between these two methods increased when the 25(OH)D concentration increased. Therefore, it can be recommended that results from DBS method should be interpreted cautiously.

Study 2 (n=20)

The findings from Study 2 were very similar to those from Study 1. As shown in **Figure 3.12** whilst estimates of 25(OH)D concentration obtained using the two methods were significantly and positively correlated (n=20, R^2= 0.517, p<0.001), values from use of DBS were consistently higher that when using the conventional approach and there was evidence of differential bias **(Figure 3.12 B)** in results from use of the two methods.



A) Regression analysis of 25(OH)D concentration (nmol/L) by conventional and DBS method



B) Bland-Altman analysis of estimates of mean 25(OH)D concentration (nmol/L) serum venous vs DBS

Figure 3. 11 (A and B): Comparison of 25(OH)D concentration using serum venous (conventional method) and DBS method.

(A), linear regression analysis between venous 25(OH)D and DBS (B), Agreement and differential bias between the two methods was evaluated by the Bland Altman method. Black horizontal lines (bold) show the mean difference between conventional and DBS method and the fine, grey line are the limits of agreement (± 2SD). A regression line was fitted to the points (dashed black line) to evaluate differential bias.

Recommendation for practice:

Similar in Study 1, the use of DBS method was found to be over-estimated 25(OH)D concentration compared with the conventional method. Furthermore, the difference between these two methods increased as the concentration of 25(OH)D increased. Therefore, results from use of the DBS method should be interpreted cautiously.

3.7.3 Discussion

In this study, large differences in 25(OH)D concentration were found between use of DBS and conventional analysis of serum samples. Analysis of DBS led to consistent over estimation of 25(OH)D concentration. By using DBS method, it seems that none of the participants in either study had vitamin D deficiency, which is doubtful.

In Study 1, the serum samples were collected with capillary blood spot at the same time from overweight and obese postmenopausal women aged 50 to 70 years old. The participants were not using vitamin D supplementation and not using artificial sunlamps (or taking sunny vacations before sampling), so we were expecting the 25(OH)D values for the participants to be in line with Northern England Data (SACN, 2016), where the seasonal 25(OH)D values are as follows:

North England (n = 676)	Mean winter 25(OH)D (nmol/L)	= 41.2 (95% CI: 39.7, 42.7)
North England (n = 1261)	Mean Summer 25(OH)D (nmol/L)	= 60.9 (95% CI: 59.5, 62.3)

Study 1 revealed the mean 25(OH)D concentration of the women using the CLIA method is 44.8 nmol/L whilst using the DBS method it is 101.8 nmol/L. Interestingly using the DBS method, none of the women would be categorized as 'vitamin D deficient' (25(OH)D < 25 nmol/L) (SACN, 2016) even though it is regularly observed that 30% of the UK population aged 50-65 and over have vitamin D deficiency throughout the year. Half of the participants have 25(OH)D > 100 nmol/L using the DBS method whereas only one participant had 25(OH)D > 100 nmol/l using the CLIA method.

In study 2, the participants were supplemented with 25 µg vitamin D for three months. The results revealed that mean 25(OH)D concentration from serum samples is 67.35 nmol/L, which is not surprising as it was as expected. However, the mean 25(OH)D concentration using DBS method was more than double (147.98 nmol/L).

A few studies have reported that use of the DBS method resulted in 25(OH)D concentrations that were substantially lower compared with venepuncture plasma/serum 25(OH)D (Eyles *et al.*, 2009; Larkin *et al.*, 2011; Heath *et al.*, 2014; Sakhi *et al.*, 2015), which is contrary to this study. A longitudinal study among 109 mother-infant dyads in Tennessee conducted by Larkin et al., 2011 demonstrated good agreement between DBS and serum. The measurement of 25(OH)D by DBS in this study were on average lower compared to serum measurement. Similarly, a cohort study done by Health et al., 2014 involving 62 participants from Melbourne Collaborative Cohort Study were done for a study calibrating 25(OH)D concentrations in DBS with plasma. The result showed good agreement between 25(OH)D from DBS and plasma. However, in this PhD study, it seems likely that the results from use of DBS are imprecise and CLIA estimations are closer to the 'true values'.

Several reasons can be postulate from this dissimilarity finding. DBS card in this study has been stored in -80ºC for about 6 months before being analysed. Even though (Heath *et al.*, 2014) in her study reported that measurements of 25(OH)D concentrations in archived DBS up to 19 years are reliable, the possibilities of some degradation of 25(OH)D might occur, which leads to inaccuracy of the results. The same condition can be observed during the drying phase of freshly spotted DBS prior to frozen storage.

The blood collection procedures, transportation and storage may lead to variation in sample quality. The compounds need to be stable in the presence of oxygen at ambient temperature for at least the initial drying time and shipment time to the laboratory. Apart from these pre-analytical procedures, biological factors such as sample viscosity and haematocrit level might be the additional challenges that lead to this discrepancy. In addition, the importance of calibration of the machines (in this case; CLIA and LC-MS/MS) prior to the measurements of the samples are essential for the reliability of the analysis. As the analysis of serum samples and DBS were analysed in Freeman Hospital, Newcastle and Birmingham Hospital, respectively, this is beyond our control, even though each of the laboratories reported that they participate in the Vitamin D External Quality Assurance Scheme (DEQAS). However, DEQAS does not operate quality control for DBS, so in this case, although the Birmingham laboratory participate in DEQAS for venous sampling, it is not applicable for DBS.

3.7.4 Conclusion

In conclusion, this study showed a large discrepancy between the measurement of 25(OH)D using conventional method (assays on liquid serum collected by venepuncture) and using a dried blood spot (DBS) method. Therefore, we ought to reject the DBS values for 25(OH)D in this study because the high results were not explained by the participant's dietary vitamin D intake (which is low and non-users of vitamin D supplements – Study 1) or sun exposure (no sunbed use or sun holidays in the previous three months prior to sampling). Furthermore, the unusually high 25(OH)D results obtained by DBS (i.e. half of the participants had 25(OH)D > 100 nmol/L) is atypical for North-East 50-70-year-old women (SACN, 2016).

3.8 Limitations in comparison and clinical interpretation of serum-levels across different assay methods - Diversity of 25(OH)D analysis perspective

In early 1970s, the first method of measuring 25(OH)D was developed using the competitive protein binding format with the vitamin D binding protein (DBP) as the binder (Wallace, 2010). Measuring serum 25(OH)D concentrations vary and it depends on the type of assay used and the specific laboratory undertaking the analysis, thus impairing the accurate assessment of vitamin D status (Lips et al., 1999).

To date, there are several commercially analytical techniques have been widely used for 25(OH)D analysis in serum or plasma, such as chemiluminescence immunoassays (CLIA), radioimmunoassay (RIA), high performance liquid chromatography (HPLC), liquid chromatography –tandem mass spectrometry (LC-MS/MS). The technical characteristics, advantages and disadvantages of each technique used for 25(OH)D analysis are described in **Table 3.7**.

Table 3. 7: The technical characteristics, advantages and disadvantages of each technique used for 25(OH)D analysis

	Method & Description	Advantages	Disadvantages
1.	*Chemiluminescence immunoassays (CLIA)* The main principle of CLIA involved the generation of electromagnetic radiation as light by the release of energy from a chemical reaction. The light is then emitted in the ultraviolet, visible or infrared region, and measured by a photomultiplier as relative light units (RLU) (Wagner *et al.*, 2009).	More practical and easier method compared to another analytical technique for 25(OH)D analysis. Higher-throughput capacity and lower sample volume requirement.	Required high skilled operator. Unable to measure $25(OH)D_2$ and $25(OH)D_3$ separately.
2.	*Radioimmunoassay (RIA)* This technique is more favour for laboratories in clinical monitoring. Developed by DiaSorin Liaison Corporation, this method was used by many reference laboratories and considered the gold standard in the past decades before the developing of LC-MS/ MS method (Hollis *et al.*, 1996).	Suitable for laboratories with a larger testing volume. More often used for clinical diagnosis due to its reliability and accuracy. Convenience and can providing fast output. Good comparability with other methods.	Unable to measure $25(OH)D_2$ and $25(OH)D_3$ separately. Involving multiple phase separation steps (washing steps), and requiring special instruments or manufacturer specific instruments to perform
3.	*High performance liquid chromatography (HPLC)* This type of assay was introduced in 1977 from the laboratory of Dr. Hector DeLuca determining $25(OH)D_2$ and $25(OH)D_3$ in human plasma using high-pressure liquid chromatography and detection by ultraviolet absorbance. This technique is more favour for laboratories carrying out research (Eisman *et al.*, 1977).	Often referred to as the standard by which other methods are compared. It can measure the individual quantitation of $25(OH)D_2$ and $25(OH)D_3$. High accuracy if validated and performed by experienced personnel.	Requires expensive equipment and highly skilled operators. The output result needs to be cautiously interpreted. The procedure need large sample volume requirements, slow sample throughput. Technically difficult, not fully automated, and of low throughputs. Unsuitable for routine as the method is very time consuming and required extensive purification steps.

	Method & Description	Advantages	Disadvantages
4.	*Liquid chromatography –tandem mass spectrometry (LC-MS/MS)* This technique is more favour for laboratories carrying out research. This method is reported to be the 'gold' standard for 25(OH)D measurement, providing the most specific and sensitive results (van den Ouweland, 2016).	It can measure the individual quantitation of $25(OH)D_2$ and $25(OH)D_3$. Highly precision and reliability. Most sensitive and specific for measuring serum 25(OH)D concentrations. Minimal adaptation to standard method required.	Need calibration to standardize the measurement. Not commonly available in clinical laboratories due to its high equipment costs. The procedure need large sample volume requirements.

3.9 Proficiency testing of 25(OH)D assays

3.9.1 Vitamin D External Quality Assessment Scheme (DEQAS)

It is well acknowledged that the measurement of 25(OH)D is challenging due to its physiological characteristic (highly lipophilic, bound strongly to DBP) (Wagner et al., 2009). Apart from that, 25(OH)D exists in two structurally similar forms, 25(OH)D_3 and 25(OH)D_2, which makes it difficult to measured. The large inconsistency and variability in 25(OH)D measurements between methods and laboratories has ignited the needs of establishment a quality assurance scheme to monitor the analytical reliability of 25(OH)D assays.

To standardize the laboratory methodology and proficiency of vitamin D assay, the Vitamin D External Quality Assessment Scheme (DEQAS) has been established to monitor 25(OH)D assay performance since 1989. Mainly operated by Charing Cross Hospital, London, UK, DEQAS has regularly conducted and reported on a number of investigations into the performance of 25(OH)D methods within laboratories worldwide (Carter et al., 2010a). Since then, more than 35 countries has participated in this scheme, as it provides expertise in vitamin D field and proficiency testing schemes and biostatistics which is beneficial for the laboratories and manufacturers (Carter et al., 2010b).

After 20 years of the establishment of this scheme, over 250 US laboratories have voluntarily registered with DEQAS and this indicates that the importance of standardization of techniques used for 25(OH)D analysis in serum or plasma has been accepted and acknowledged (Hollis, 2008). In April 2013, DEQAS has been collaborated with US National Institute of Standards and Technology (NIST). From this collaboration, each of the DEQAS samples will have the values assigned by the NIST Reference Measurement Procedure (RMP) for the individual metabolites 25(OH)D_3, 25(OH)D_2 and additional 3-epi – 25(OH)D_3, which is significant for laboratories using HPLC/UV and LC-MS/MS methods (Vitamin D External Quality Assessment Scheme, 2014).

Apart from the collaboration with NIST, DEQAS is also mutually participating with Vitamin D Standardisation Program (VDSP). VDSP was established in 2010 by the US Office of Dietary Supplements (ODS) and the objectives are similar to DEQAS, which is focusing in the standardization of laboratory measurement of 25(OH)D. This scheme also conducting

international research programs which is committed to improve the quality of laboratory measurement of 25(OH)D (Vitamin D External Quality Assessment Scheme, 2014).

The combination of these three quality assurance scheme (DEQAS, NIST and VDSP) has bring new important contribution for the analytical reliability of 25(OH)D assays, which is very important for the integrity of the laboratory performance (Vitamin D External Quality Assessment Scheme, 2014).

Chapter 4: Association of Vitamin D Status with Physiological and Biochemical Markers of Endothelial Function in Overweight and Obese Post-Menopausal Women

4.1 Introduction

Vitamin D, the 'sunshine vitamin', is currently the subject of controversy in relation to its role in cardiovascular disease (CVD) prevention. Primarily known for its key role in calcium homeostasis and in bone health, there has been increasing study of the actions of vitamin D on non-skeleton health. The latter studies have been stimulated by evidence from epidemiological studies (Judd *et al.*, 2008; Anderson *et al.*, 2010; Bajaj *et al.*, 2014) and following the discovery that vitamin D regulates the expression of a very large number of genes involved in multiple cellular processes related to CVD (Dobnig *et al.*, 2008; Jablonski *et al.*, 2011; Wang *et al.*, 2013).

Investigations of links between vitamin D intake/status and CVD risk have yielded heterogeneous results which appear to be related to the study methodology. Most observational studies suggest a positive link between higher vitamin D intake/status and cardiovascular health, e.g. risk of hypertension is increased in those with low serum 25(OH)D concentration in blood (Judd *et al.*, 2008; Rueda *et al.*, 2008; Chan *et al.*, 2012). Similarly, a study from the US showed that lower serum 25(OH)D concentrations were associated with highly significant increases in the prevalence of diabetes, hypertension, hyperlipidaemia, and peripheral vascular disease (Anderson *et al.*, 2010). This is in line with a study by Wang et al, 2008, which demonstrated that serum 25(OH)D concentrations were inversely associated with risk of coronary artery disease, myocardial infarction, heart failure and stroke (Wang *et al.*, 2008). Details of the findings from observational studies on vitamin D and CVD related outcomes are summarised in **Appendix 4.1**.

Results from randomized controlled trial (RCTs) demonstrated inconsistent results. Some RCTs reported a positive effect of vitamin D supplementation on surrogate markers of vascular disease. For example, when assessing effects of serum 25(OH)D concentration on endothelial function, some studies have seen improvements in endothelial function (Tarcin *et al.*, 2009; Dong *et al.*, 2010; Harris *et al.*, 2011a) while other RCTs reported negative results (Witham *et al.*, 2010; Gepner *et al.*, 2012; Stricker *et al.*, 2012). For blood pressure, some, but not all studies have reported a positive effect of vitamin D supplementation on blood pressure

(Margolis *et al.*, 2008; Sugden *et al.*, 2008; Nagpal *et al.*, 2009; Zittermann *et al.*, 2009). Several trials found that there were no effect of vitamin D supplementation on glycemia or incident of diabetes (Hsia *et al.*, 2007; Pittas *et al.*, 2007a; de Boer *et al.*, 2008; Jorde and Figenschau, 2009; von Hurst *et al.*, 2010). Therefore, the available data are insufficient to make a conclusion about the effects of vitamin D status (as assessed by 25(OH)D concentration) on risk of cardio metabolic outcomes.

In Chapter 2, the findings from RCTs on effects of vitamin D supplementation on endothelial function have been discussed. The results of that systematic review and meta-analysis were mainly equivocal. In this chapter, the findings from a cross sectional study will be described and discussed which investigated the association of serum 25(OH)D status with endothelial function (EF) in older overweight and obese, postmenopausal women who are at higher CVD risk. Such an association may be explained by the link between serum 25(OH)D concentration and pathways involved in the regulation of nitric oxide (NO) signalling and EF.

Serum 25(OH)D concentration may influence NO and EF indirectly by suppressing renin angiotensin aldosterone (RAS), a hormone that control the regulation of blood pressure (Li et al., 2002), and, in particular, low circulation of serum 25(OH)D may increase the expression of RAS. Reduction of PTH is also associated with high circulating concentrations of serum 25(OH)D concentration (Yankouskaya and Snezhitskiy, 2014). Higher PTH concentrations are associated with increased risk of CVD events by increasing blood pressure, cardiac hypertrophy, and myocardial dysfunction (van Ballegooijen et al., 2013). Therefore, the rationale of the cross sectional study reported in this chapter is that those with higher serum 25(OH)D concentrations group will have lower PTH. The vitamin D receptor (VDR) is expressed in smooth muscle cells (SMC) and endothelial cells, which strengthens the argument that serum 25(OH)D is involved in maintenance of the integrity of EF perhaps by enhancing the proliferation SMC and through expression of vascular endothelial growth factor (VEGF) (Kamen and Tangpricha, 2010; Ferder *et al.*, 2013; Min, 2013). A stronger association between serum 25(OH)D concentration and physiological markers of EF (PORH and PWV) would be predicted as these markers provide an integrated assessment of various pathways involved in the control of vascular homeostasis. Therefore, we speculate that there will be an inverse association between serum 25(OH)D concentrations and measures of EF and blood pressure.

4.2 Hypothesis, aim and objectives

The main hypothesis of the project was based on the occurrence of a higher prevalence of vitamin D deficiency in individuals with signs of endothelial dysfunction (Alyami *et al*., 2014). Specifically, we evaluated to test whether lower levels of serum 25(OH)D concentration were significantly associated with greater impairment of EF, assessed using physiological and biochemical biomarkers in older, overweight and obese postmenopausal women.

The primary aim was to conduct a cross-sectional study including 80 middle-aged and older, overweight and obese post-menopausal women to investigate the association between serum 25(OH)D concentration, physiological and biochemical markers of EF.

The specific objectives of the study were:

1. To collect socio-demographic, dietary, lifestyle information and assess body composition and resting blood pressure.
2. To investigate the association between serum 25(OH)D concentrations and PORH and PWV, measured by laser Doppler and digital photopletysmography (PPG), respectively.
3. To evaluate the association between serum 25(OH)D concentrations and biomarkers of EF including asymmetric dimethylarginine (ADMA), cyclic Guanosine Monophosphate (cGMP), Endothelin-1 (ET-1) and Vascular Endothelial Growth Factor (VEGF).

4.3 Methods

4.3.1 Study design

This was a cross sectional study involving healthy, overweight and obese post-menopausal women living in Newcastle and nearby. The study was conducted in the Clinics for Research and Service in Themed Assessment (CRESTA) facility, Newcastle Biomedical Research Building on the Campus for Ageing and Vitality, Newcastle University. The study took place between December 2014 and August 2015.

4.3.2 Participants recruitment

This study was aimed to recruit volunteers from the general public. A total of 80 healthy (with no established medical diagnosis), non-smoking, overweight or obese, post-menopausal women with a BMI between 25.0 and 40.0kg/m^2, and age range between 50 to 70 years old

were recruited from the general public in Newcastle through volunteer databases available at Newcastle University, circulation of emails to members of staff of Newcastle University and Newcastle NHS Hospitals, dissemination of posters and leaflets as well as advertisement in the local community in public places including the City Library and local shops . We also sought support from Voice North (the regional research and engagement panel (http://www.ncl.ac.uk/iah/ageing/volunteer/voicenorth.htm). Each advertisement contained concise information on the study title, aims of the study, main inclusion criteria (age, BMI, gender, smoking status) and contact details of the study coordinators.

Telephone screening

Telephone screening was conducted to check participant eligibility. A standardised questionnaire was used to ensure the accurate evaluation of the inclusion and exclusion criteria as well as to collect information on sociodemographic status, GP practice, smoking behaviour and commitment and availability to complete the study. Participants were excluded if they have any of the following criteria: weight change more than 3.0 kg in the last three months; took nutritional supplements which provided calcium (dosage more than 500 mg/day) and vitamin D (dosage more than 10 mcg per day - this included those on vitamin D preparations for joint pain either prescribed by their GP or obtained over the counter); diagnosis of cancer and any diagnosis of malignant cancer in the last five years; chronic and acute metabolic and inflammatory conditions which could interfere with the study outcomes; previous diagnosis of type 1 diabetes or type 2 diabetes treated with insulin; use of weight loss medication (sibutramine, orlistat or rimonabant) or history of bariatric surgery; use of specific drugs including: oral corticosteroids, anticoagulants, nitrate-derived agents, anti-cholinergic drugs; use of hormonal therapies (oestrogens, thyroxine, progesterone, oral hypoglycaemic agents), anti-hypertensive (diuretics, beta-blockers, calcium antagonists, ACE-inhibitors and angiotensin receptors inhibitors), statins and any other anti-dyslipidaemic agent and use of psychiatric drugs (antidepressants, sedatives, antipsychotics) if this use had started, or the dose had been changed, in the previous three months; haematological disorders including previous diagnosis of severe anaemia (Hb < 10 mg/dL); major surgical operations interfering with the study outcomes; alcohol intake >14 units/week; non English speakers or volunteers requiring translators or interpreters.

During the telephone screening, the participants were asked about their contact details (home address, email address, telephone number (home/office/mobile), name and address of medical

practice, anthropometric measurements (weight and height), health status, medication that they consumed (prescribed or over the counter) smoking and drinking status. If they were eligible and willing to participate in the study, appointments were set up based on the availability of the potential participants to attend and the availability of the clinic.

Study invitation

An invitation email was sent to the potential participant for the confirmation about the study visit. The email contains the date, time and venue of the study visit. The participant information sheet also was send as an attachment in the email for them to read and have a brief idea about the study. For those without email address, an invitation letter was sent by mail.

Two days before the study visits were held, a reminder email was sent to the participant and details of the route to the CRESTA clinic were attached. For those who needed transport to come to the clinic, a taxi was booked for them and details of the booking were sent by email. The participant was reminded to be fasting starting from 8.30 pm on the day before the study visit (water was allowed). Since they were required to lie down during the visit for the vascular measurement, the participant was advised to wear comfortable clothes (a short sleeve top/blouse and trousers rather than a long sleeves or a dress).

From December 2014 to April 2015, we conducted one session of study visits every Monday, Tuesday and Friday (one participant per session). From May to August 2015, we conducted two sessions of study visits (two participants per day). For the latter, session one ran from 8.15 am to 9.45 am and session two from 9.30 am to 11.00 am.

Equipment set up

Before the study visit, all the forms necessary for the participant's visit were printed and organized in a folder (screening form, consent forms, study form, information sheet, study questionnaires, tracking form, reimbursement form and equipment manual). A request form was send to Clinical Ageing Research Unit (CARU) to assess the participant's clinical note three days before the study visit was held.

Labels and tubes for the collection of blood and urine samples were prepared a day before the study visit. This included labelling the tubes for storage of aliquots of blood and these tubes

were kept in the laboratory until required. The equipment for dry blood spot collection were prepared (lancet, Protein Saver^{TM} 903^{TM} card, alcohol swab, plaster).

Equipment for vascular measurements (laser Doppler and PWV) were set up to ensure the effectiveness of the measurement during the study visit. The weighing scale and portable blood pressure device were tested for the battery efficiency. A breakfast voucher and honorarium voucher were prepared in an envelope for the participant.

4.3.3 Sample size

The calculation of the sample size was based on the results of a cross sectional study which investigated differences in FMD in middle aged/older men and women stratified by vitamin D status (deficient, normal). This study found a significant difference in FMD between the groups with the following results: Normal: 4.75±19.2%, n=22; Deficient: 3.33±1.87%, n=22 (Jablonski *et al.*, 2011). We used this information to estimate the sample size required to detect a significant effect in vascular measurement between obese post-menopausal women with normal and those with deficient vitamin D status. The calculations showed that 58 overweight and obese post-menopausal women will be required to detect a significant difference between the two groups with an α level of 0.05 and a power (1-β) of 0.80. We aimed to recruit 80 participants for this study. The calculations were performed using G power 3.1 for Windows. FMD was used as the method to estimate sample size even though this study used PWV and PORH for the assessment of EF. This was because at the time of the design of the study, no study was conducted using PWV/PORH for the measurement of EF and association with serum 25(OH)D concentration. Therefore, the magnitude of the difference in FMD observed by Jablonski et al, 2011 was assumed and applied to this study.

4.3.4 Study sponsorship

The Newcastle upon Tyne Hospitals NHS Foundation Trust acted as the research sponsor for this study, under the Department of Health's guidelines for research in health and social care (Trust R&D Project No: 7074) **(Appendix 4.2)**.

4.3.5 Ethical approval

REC application

Prior to recruiting any participants, ethical approval was sought and gained from NRES Committee East Midlands-Northampton (14/EM/1073) **(Appendix 4.3)**.

Substantial amendment

A substantial amendment to the ethical approval was sought and obtained to amend the exclusion criteria about the permitted dosage of nutritional supplement (specifically vitamin D and calcium) consumed by the participants **(Appendix 4.4)**.

R&D application

IRAS project ID: 152807

Caldicott and Data Protection approval for storage of data was sought and gained from The Newcastle upon Tyne Hospitals NHS Foundation Trust (3331).

4.3.6 Clinical assessment

Participants arrived at Clinics for Research and Service in Themed Assessment (CRESTA) early in the morning (~8.30 am) after an overnight fast of about 12 hours and were asked to avoid caffeinated drinks before the visit.

Participants were provided with a Participant Information Sheet which outlined the aim of the study and what they would be required to do. The potential risks and benefits of their participation were clearly explained in the information sheet and subjects were informed that, while their anonymity would be preserved and their data stored safely, the group data may be published in academic journals or presented at national/international conferences. Participants were told that they were free to withdraw from the study at any point with no disadvantage to themselves. Any additional questions or concerns that the participants had were answered before they provided written, informed consent to participate.

Measurements

Anthropometric measurements

Height and weight were measured with a standard stadiometer and weight scale, with participants wearing light clothing and barefooted. Weight was measured in kilograms to the nearest 100g. Weight and height measurements were used to calculate BMI according to the following equation:

$$\text{BMI} = \frac{\text{weight (kg)}}{\text{height (m)}^2}$$

Waist circumference was measured with a standard non-stretchable tape, in the standing position, at the minimum circumference between iliac crest and the rib cage. Body composition was measured using a leg-to-leg bioelectrical impedance device (TANITA 300 MA).

Blood pressure measurement

Blood pressure measurement was done in triplicate at one minute intervals using an automated blood pressure monitor (model: Omron M3 [HEM-7200-E8(V)]) with the participant in the sitting position. Before the measurements, participants were invited to rest for a minimum of five minutes. An appropriate cuff size was used for each subject.

Biochemical measurements

A total of 20 ml fasting venous blood were drawn by venepuncture in the antecubital fossa and transferred into five different vacutainers (lithium heparin, serum gel, EDTA, plain tube, sodium fluoride). Blood samples were separated by centrifugation within two hours of being drawn and aliquots were stored at -80ºC until analysis. The EDTA vacutainer were sent to Clinical Biochemistry Laboratory (Freeman Hospital, UK) on the same day for the analysis of haematocrit.

A finger prick capillary blood samples was obtained for vitamin D concentration using the dried blood spot approach. The blood drops then were transferred onto a specific filter paper (blood spot card) (Protein Saver™ 903™ Card, DSM). Blood spots were air dried for at least 30 minutes before the flap was closed on the blood spot card. Fully dried samples were then stored at -80ºC until required for vitamin D assay. A spot urine sample was collected in urine plastic container.

Vascular measurement

The final part of the visit was the assessment of vascular health. During the measurements, participants were asked to lie on a bed for 30 minutes and to avoid any movement as this may affect the validity of the measurements. The first test was called "Pulse Wave Velocity" which measures how fast the heart beat travels in the arteries **(Table 4.1)**. This test is simple, non-invasive, validated and reproducible measurement of arterial stiffness (Laurent *et al.*, 2006). The stiffer the arteries are, the faster the wave is returned, adding to the forward wave and

augmenting the systolic pressure (augmentation index (AI)) (Cho *et al.*, 2013). Developed by the Medical Physics Department of Newcastle University, photoplethysmography (PPG) was developed to measure the PWV and distensibility coefficient (DC). The procedure involved the attachment of five electrodes using small adhesive gel pads to the ear lobe, fingertip, chest and abdominal area. The data were recorded and analysed by a computer (Fujitsu Lifebook S3020 laptop, Japan). Single lead ECG was also recorded simultaneously **(Figure 4.1)**. A series of two separate measurements were performed with different external cuff pressures (0 and 40 mmHg) applied to the whole right arm through the long cuff, with a minute rest interval between each measurement. During the recording session, the arms were kept parallel to the body and as still as possible, with regular and gentle breathing. The measurement last approximately 15 minutes. The data, display, ECG and PPG signal acquisition were stored in software named ARD (Analogue Recorder and Display), developed by the Medical Physics Department.

Table 4. 1: Photoplethysmography variables

Variables	Definitions
1. Pulse Transit Time (ms)	The time it takes for the pulse pressure wave to go through an arterial tree in the arm, resulted from the ejection of blood after the release of the 40mmHg pressure applied to the arm.
2. Pulse Wave Velocity (m/s)	The distance travelled by the wave **(Δx)** divided by the time for the wave to travel for that distance **(Δt)** when 40mmHg pressure was applied to the arm. $\frac{(\Delta x)}{(\Delta t)}$

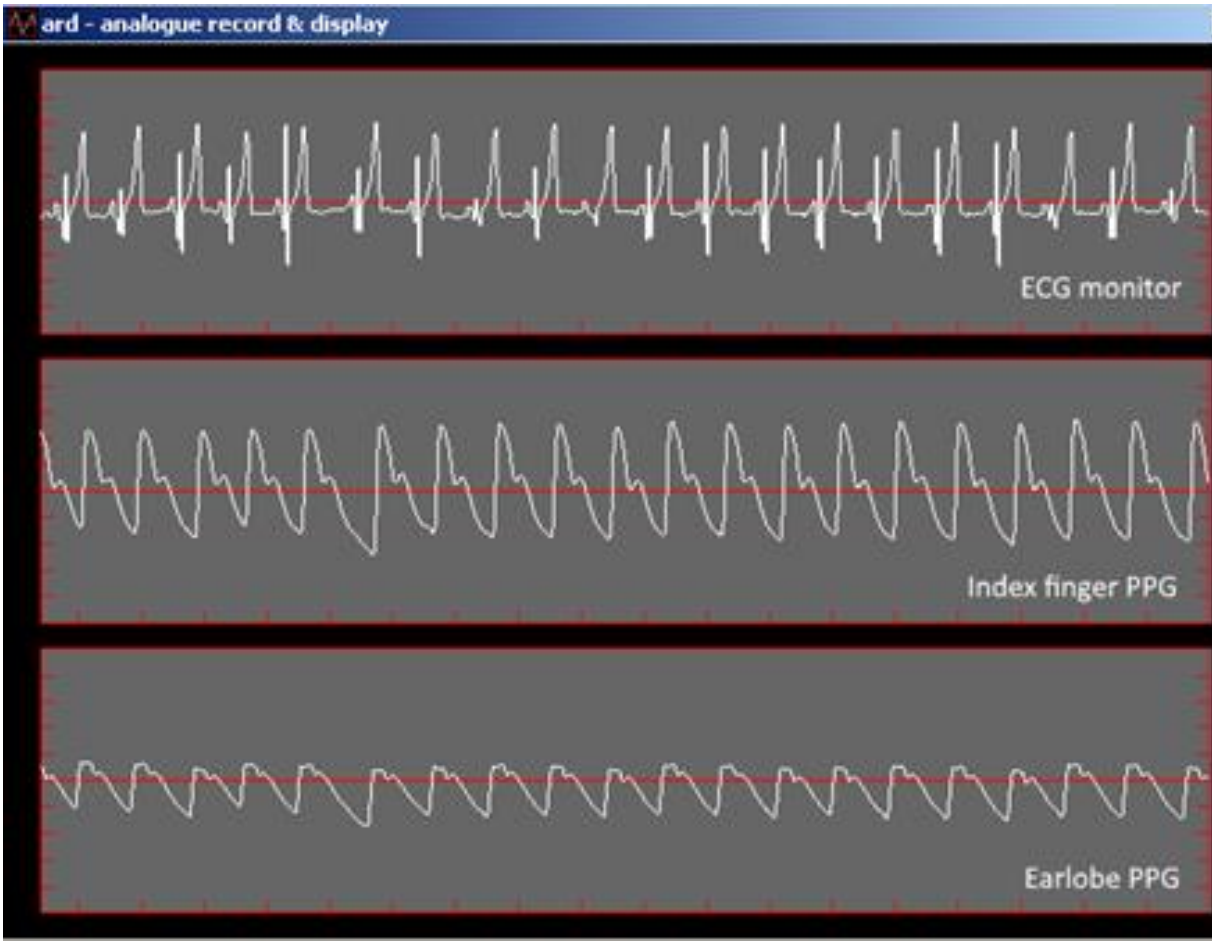


Figure 4. 1: Example of pulse wave recording of study participant.

At the end of the test, the electrodes were removed and the second test was performed, which is called Post- Occlusive Reactive Hyperaemia (PORH) **(Table 4.2)**. This test was used to measure the cutaneous microvascular reactivity and based on the ability of the endothelium to release nitric oxide (NO) as a response to proximal arterial occlusion. After the end of the occlusion, when the peripheral perfusion is restored, the immediate reduction of vascular resistance results in an increase of the blood flow which is closely linked to NO release and integrity of endothelial function (Carasca et al., 2017). The duration of the test was approximately 15 minutes. A laser Doppler probe was attached to the right forearm. The test involved the placement of a blood pressure cuff around the participants' arms which were inflated for three minutes to 200mmHg to obstruct the blood flow in the brachial artery. After the deflation of the pressure cuff, the hyperaemic response was recorded using Moor VMS V3.1 software. PORH index was calculated as the ratio between Area Under Curve (AUC) a minute after the deflation of the cuff with AUC of a minute before the inflation of the cuff **(Figure 4.2** and **Figure 4.3)**.

Table 4. 2: Post-Reactive Occlusive Hyperaemia variables

	Variables	Definitions
1.	Resting level (RL)	The first three minutes of the procedure.
2.	Maximum level (ML)	The maximum level of the graph of the blood flow after the release of the cuff at 200mmHg.
3.	Area of occlusion (AO)	The area when the occlusion happens and blocks the blood flow (shown as blue area in the graph).
4.	Area of hyperemia (AH)	Area under the curve after the release of the cuff (shown as pink area in the graph).
5.	Area of hyperemia (AH) / area of occlusion (AO)	Area under the curve after the release of the cuff divided with area when the occlusion happens and block the blood flow.
6.	Time to recovery (TR)	The first few second after the release of the cuff.
7.	Time to maximum (TM)	The time taken to reach the peak of the graph of hyperemia.
8.	Time to half decay from maximum (TH)	The time taken to reach the half of area of hyperemia.
9.	PORH Index	$\frac{\text{Area under curve one minute after the deflation of the cuff}}{\text{Area under curve one minute before the inflation of the cuff}}$

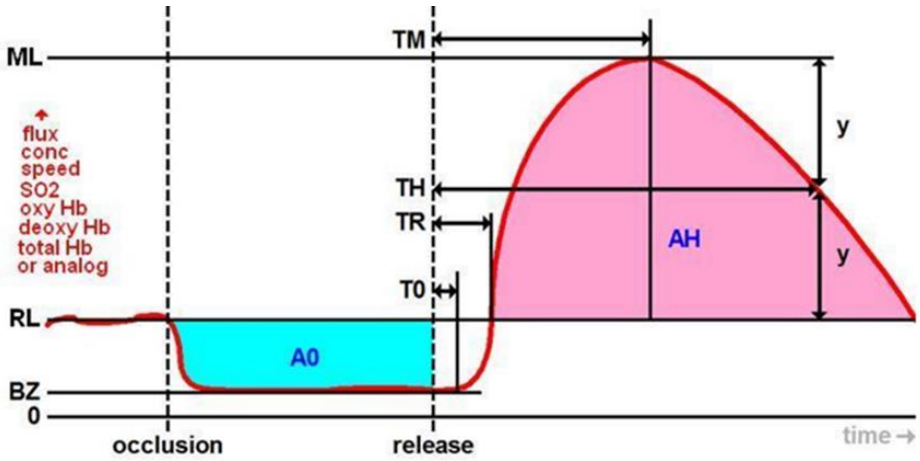


Figure 4. 2: Graphical statistic of PORH analysis

(Taken from moorVMS recording and analysis software V4.0, Vascular Monitoring System Software Manual)

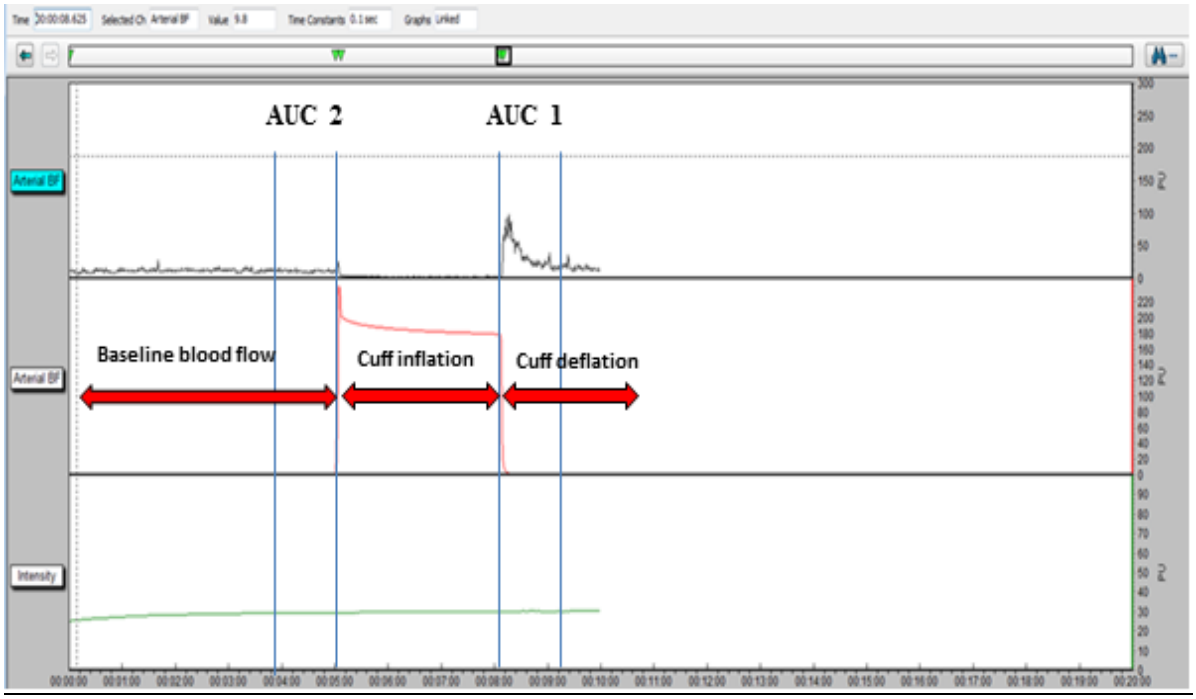


Figure 4. 3: Laser Doppler Iontophoresis (LDI) recording from a participant during one of the study visits.

Questionnaires

Participants were asked to complete a battery of questionnaires regarding their general health, lifestyle and physical activity **(Appendix 4.5)**. In addition, participants' diets were assessed using a self-administered Food Frequency Questionnaire (FFQ) **(Appendix 4.6)** for the assessment of overall dietary and nitrate intake. The intakes of energy and nutrients were calculated using the MEDE FFQ Database. Short questionnaires on sun exposure were used to evaluate the participant's overall sun exposure **(Appendix 4.7)**.

This completes the study visit. At the end of the visit participants were provided with an honorarium of £10, a £3 voucher for light refreshment at the cafeteria and reasonable travel expenses were also been paid to each participant. The quantitative measurements made on each participant during the study visit were summarised in **Table 4.3**.

Table 4. 3: Table summarising the measurement method, equipment and duration of the quantitative measurements taken during the cross-sectional study visit

Outcome	Equipment	Procedure	Approximate time taken to complete
Weight (kg), BMI, Body Fat%, Fat Mass (kg)	Bioelectrical impedance device (TANITA 300 MA).	Participants were asked to stand barefoot on the scale. Age, body type, gender and height were imputed. Results for anthropometric measures are printed and recorded in the data collection sheet.	5 minutes
Height (cm)	Stadiometer	Participants were asked to stand straight with their back towards. Stadiometer ruler and the marker were brought down to the top of the head. Height was measured from the marker and measured 3 times.	5 minutes
Waist circumference (cm)	Flexible measuring tape (marked in centimetres)	Waist size was measured using a measuring tape halfway between the bottom rib and iliac crest.	5 minutes
Blood pressure measurement (mmHg)	Automated blood pressure monitor (Omron M3 (HEM-7200-E8(V)))	The participant's upper arm was placed with the BP cuff and measured in triplicate.	5 minutes
Serum 25(OH)D concentration, Serum Calcium and Haematocrit	1x Plain, 1x Serum Gel, 1x EDTA, 1x Sodium Fluoride, 1x Lithium Heparin Vacutainer Tubes, Butterfly needle, vacutainer tube holder, antiseptic wipe, tourniquet, gauze, plaster, pathology sample bag	The participant's arm is assessed and prepared before blood is taken from the vein, using a vacutainer blood collection system.	10 minutes

Outcome	Equipment	Procedure	Approximate time taken to complete
Serum 25(OH)D concentration	DBS (Dried Blood Spot) Kit: containing DBS card, Foil packaging, high flow lancet, plaster, antibacterial wipes and sterile gauze.	The participants washed their hands thoroughly. The researcher disinfected finger before blood is obtained by finger prick. Blood drops are placed on a filter paper and dried at room temperature.	5 minutes
Vascular measurement		Participants were asked to lie on a bed for about 25-30 minutes and avoid any movement. All the measurements were taken in a temperature controlled room (~23 ± 1C).	
• Pulse Wave Velocity	Small adhesive gel pads, computer (Fujitsu Lifebook S3020 laptop, Japan), 5x optical probes, single lead ECG, long blood pressure cuff	The procedure involved the attachment of five optical probes using small adhesive tape to earlobe, fingertip, chest and abdominal area. A long cuff was wrapped around the whole arm of the participants and a series of two separate measurements was performed with different external cuff pressures (0 and 40 mmHg) with a minute rest interval between each measurement. Single lead ECG was recorded simultaneously. During the recording session, the arms were kept parallel to the body and as still as possible, with regular and gentle breathing. The information was recorded and analysed by a computer.	15 minutes
• Post-Reactive Occlusive Hyperaemia	Laser Doppler probe, Computer, blood pressure cuff, Moor VMS V3.1 machine and software	A small probe was attached to the forearm. The test involved the placement of a blood pressure cuff around the participant's arm which was inflated for three minutes to 200mmHg.	15 minutes

Outcome	Equipment	Procedure	Approximate time taken to complete
Questionnaires	• FFQ • Nitrate intake • IPAQ • Sunshine exposure questionnaires	Participants were asked to complete a battery of questionnaires regarding their general health, dietary pattern, lifestyle and physical activity.	30 minutes

4.3.7 Participant Feedback

On conclusion of the study, each participant was mailed a letter thanking them for their participation in the study and an individual feedback sheet which provided data collected during their study visit. This included:

- BMI (kg/m^2)
- Waist measurement (centimetres)
- Blood Pressure- systolic and diastolic (mmHg)
- Blood Vitamin D (25(OH)D) concentrations (nmol/L)
- Blood Calcium concentrations (mmol/L – data were interpreted as normal, low and high)

4.3.8 Sample storage

Blood samples for serum or plasma collection were spun in a centrifuge (Jouan CR3i) at 3000 rpm for 10 mins at 4°C. Using a Gilson pipette, plasma/ serum was taken off the spun samples, aliquoted into pre-labelled aliquot tubes and stored in the -80ºC freezed at the Human Nutrition Research Centre (HNRC) as quickly as possible for further analysis. A fresh pipette tip was used for each sample tube. The labelling on each aliquot tube was checked to ensure that it matched the labelling on the sample tube. Used pipette tips and recapped discarded sample tubes were disposed in the biohazard bins. Plasma from serum gel and EDTA tube were transfer into aliquot tubes and were sent to the Freeman Hospital for 25(OH)D, PTH and calcium analysis. One EDTA tube was send to Freeman Hospital on same day for the haematocrit analysis.

4.3.9 Statistical analyses

Baseline characteristics of the participants are presented as arithmetic means ± SDs for numerical variables and counts and percentages for categorical variables. We tested the assumption of normality for all variables by testing the data graphically (visual inspection on the histogram) and numerically (by looking at the p-value for the Shapiro-Wilk, if the p-value < 0.05, the data are considered as not normally distributed, and vice versa). For non-normally distributed variables, we applied logarithmic transformations and data are presented as median (with 25^{th} and 75^{th} percentiles). Mean concentrations of serum 25(OH)D were computed and aggregated into two groups i.e. below and above the cut-off for adequacy (<30 nmol/L and ≥30 nmol/L). Pearson correlation analysis was used to assess

correlations between pairs of variables. Independent sample *t*-test was used to compare between two groups of variables. Multiple linear regression models were used to examine the associations between PWV (independent variable) and various clinical measures (e.g. 25(OH)D concentration, cGMP, ADMA, age, BMI and PAL) as dependent variables. We also derived the EF *z*-score as the mean of the following six standardized continuous indexes of NO_3^-, cGMP, ADMA, 3NT, VEGF and ET-1. To calculate the *z*-score, the difference between values in the indexes and the mean were divided by the standard deviation. As the biomarkers carries different values (cGMP, VEGF and NO_3^- were considered as positive) and (ADMA, 3NT and ET-1 were considered as negative values) for the EF, a careful consideration was given to the direction of the association between biomarkers of EF. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 22.0 program for Windows (SPSS Inc., Chicago, IL). Two sided $p<0.05$ was considered statistically significant in all cases.

4.4 Results

4.4.1 Participant recruitment

Two hundred letters were sent out to potential participants obtained from the volunteer database. A total of 99 participants were contacted for the screening interview. After screening, 10 subjects did not meet the criteria (not eligible due to health condition: five subjects, BMI<25 kg/m^2: three subjects, reluctant to draw blood: one subject, location too far from the research centre: one subject). Seven individuals dropped out after being given the appointment date due to change of mind and two individuals did not attend the research centre on the date of appointment. Hence, 80 participants completed the study **(Figure 4.4)**.

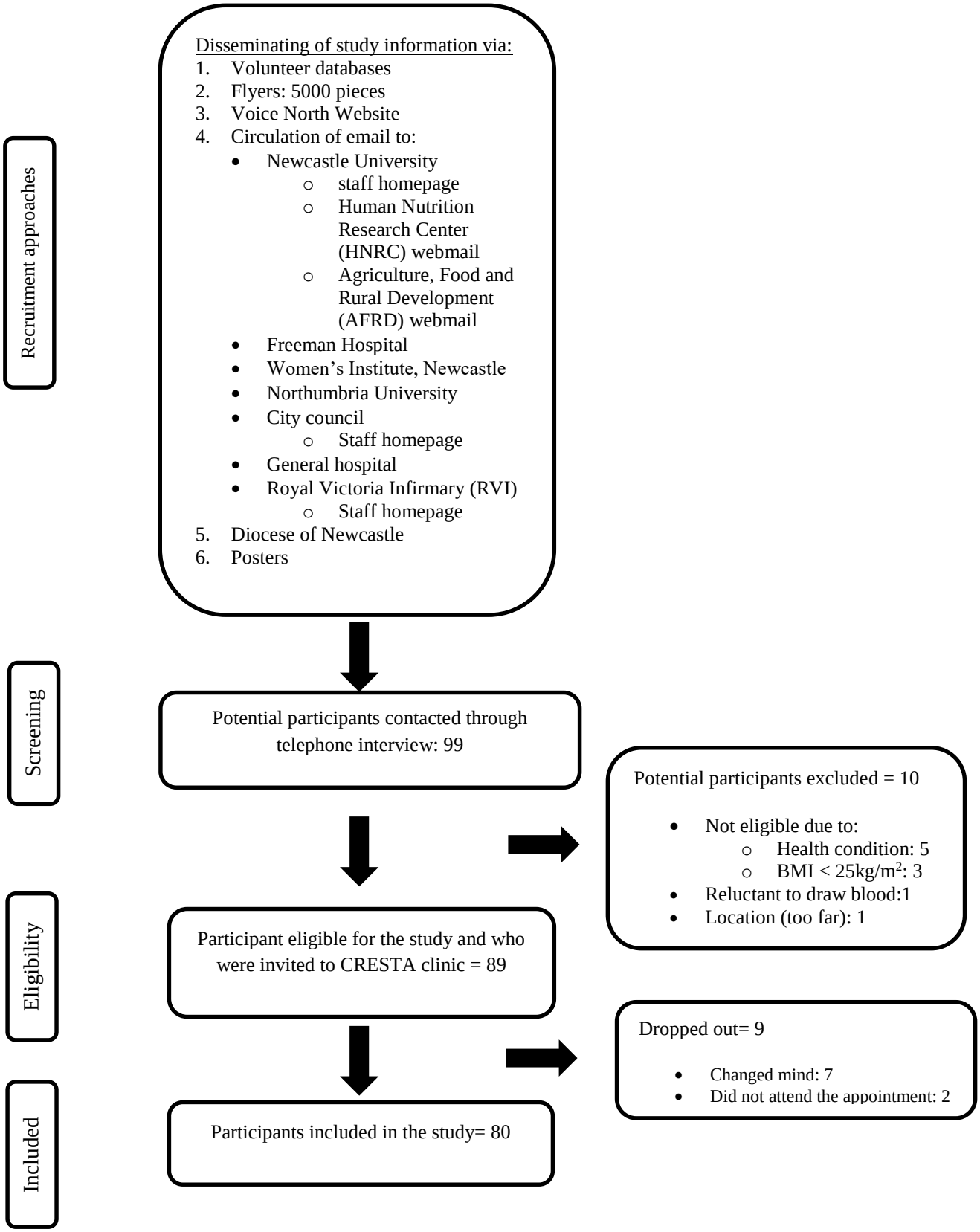


Figure 4. 4: Recruitment flow chart

The duration of this study was eight months: recruitment started on 16th December 2014 and the last participant was recruited on 11th August 2015. Due to a protracted ethical approval process, this study which was designed to start in September 2014 was delayed until December 2014. The eligibility requirements also contributed to the delay in completing the study because it was challenging to find potential participants in the appropriate age range (50 to 70 years old) who did not take nutritional supplements containing calcium and/or vitamin D. Therefore, a substantial amendment was submitted to the ethics committee to permit inclusion of those taking low doses of calcium (not more than 500 mg/day) and/ or vitamin D (not more than 10 mcg per day).

4.4.2 Baseline characteristics of participants

The characteristics of the study participants are summarized in **Table 4.4**. Mean age of participants was aged 60.0 (IQR 57.0, 67.7) years, all were overweight or obese with mean BMI 28.9 (IQR 25.9, 32.0) kg/m^2, and their mean BP was normal; mean systolic BP (SBP: 129 IQR 121.0, 142.5 mmHg), mean diastolic BP (DBP: 74.6 ± 9.5 mmHg). However, when individual measures of BP were examined, 28.4% of the participants were categorised as having high blood pressure (SBP >140 mmHg and DBP > 90 mmHg), and three participants (3.7%) were having low blood pressure (DBP <60 mmHg).

For the biochemical measurements, the mean 25(OH)D concentration was 42.5 nmol/L, the mean calcium concentration was 2.0 mmol/L, which is classified as normal (range between 2.0-2.6 mmol/L) and the mean PTH concentration was 4.5 pmol/L (normal range of PTH between 1.2 - 5.8 pmol/L). The average age at menopause was 50.0 years and 10% (8/80) had an early menopause, defined as occurring before the age of 45 years.

Table 4. 4: Characteristics of study participants

	Mean (SD)	Min - Max
Age (years)*	60.0 (57.0, 67.7)	50.0 - 70.0
Weight (kg)*	73.1 (66.9, 83.7)	53.0 - 115.2
Height (cm)	160.2 (6.3)	146.0 - 177.0
BMI (kg/m^2)*	28.9 (25.9, 32.0)	24.7 - 42.9
FM (kg)	31.2 (10.3)	18.0 - 53.4
SBP (mmHg)*	129.5 (121.0, 143.5)	96.0 - 212.0
DBP (mmHg)	74.6 (9.5)	50.0 - 101.0
HR (bpm)	65.9 (9.9)	43.6 - 92.3
25(OH)D (nmol/L)*	42.5 (26.2, 60.7)	12.0 - 125.0
Calcium (mmol/L)	2.0 (0.1)	1.8 - 2.3
PTH (pmol/L)*	4.5 (4.0, 6.1)	2.0 - 13.0
RBC (n $x10^{12}$/L)	4.5 (0.3)	3.7 - 5.3
WBC ((n x 10^9/L)	5.5 (1.6)	3.1 - 13.8
Hb (g/L)	133.9 (9.5)	109.0 - 157.0
Platelets (n x 10^9/L)	256.0 (50.0)	88.0 - 402.0
Age at menopausal (years)*	50.0 (47.2, 52.0)	40.0 - 60.0

*Reported as median (25^{th}, 75^{th} percentiles)
BMI, Body Mass Index; FM, Fat Mass; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; HR, Heart Rate; PTH, Parathyroid Hormone; RBC, Red Blood Cell; WBC, White Blood Cell; Hb, Hemoglobin.

Most of the participants were recruited in summer (Jun to August; 46%), followed by spring (March to May; 36%) and winter (December to February; 18%). Almost 60% of the participants had a family history of heart disease, followed by stroke (46.3%), diabetes (41.3 %) and lung disease (40.0%) **(Figure 4.5)**.

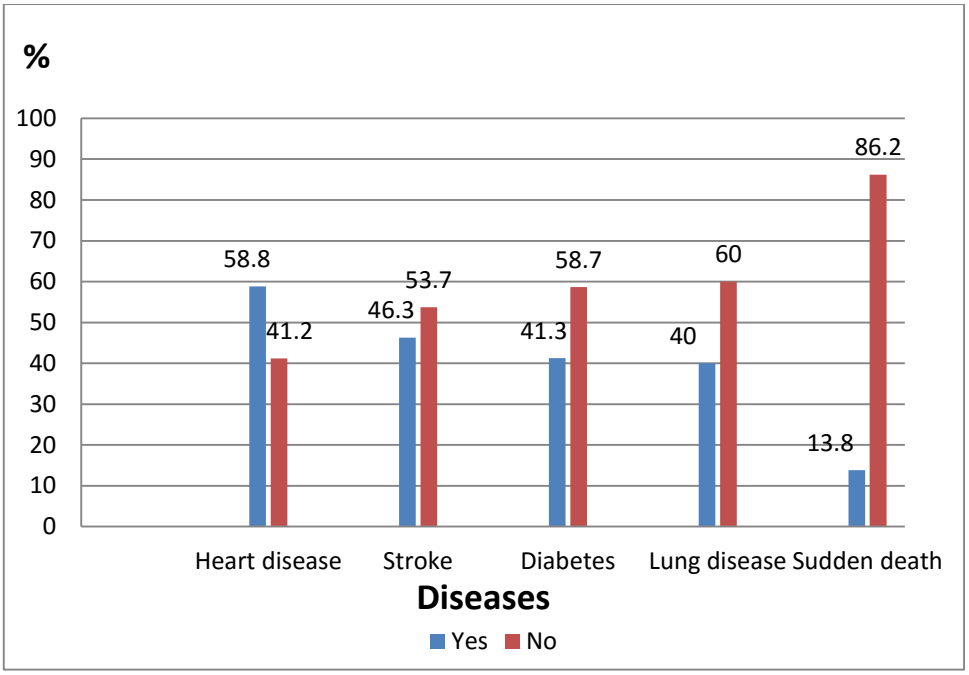


Figure 4. 5: Family history of with diseases among participants

When stratified by serum 25(OH)D concentration into two groups (<30 nmol/L and ≥30 nmol/L), there was no significant difference in the proportion of the participants with lower vitamin D status who were recruited in winter and spring (December 2014 to March 2015) compared with those participants recruited in summer (April to August 2015). However, majority of the participants (65%) recruited in winter and spring had serum 25(OH)D concentration less than 30 nmol/L **(Table 4.5)**.

Table 4. 5: Relationship between season of recruitment and vitamin D status

25(OH)D concentration	Season		P value
	Winter and spring N (%)	Summer N (%)	
• <30 nmol/L	28 (65)	26 (70)	0.75
• ≥30 nmol/L	15 (35)	11 (30)	

Analysis has been conducted using chi-square test.

There was no significant difference in mean age, anthropometric measurements (weight, height, BMI, fat mass) and physical activity levels between the two groups of participants stratified by vitamin D status **(Table 4.6)**. There was no significant difference in mean intakes

of nutrients between these two groups except for - vitamin B12 and vitamin D intake ($p<0.05$). Those participants with higher vitamin D status (≥30 nmol/L 25(OH)D) had higher intakes of vitamin B12 (5.1 vs 3.7 mg/day) and for vitamin D (3.6 vs 2.7 µg/day). NB These intakes of vitamin D from all sources are considerably less than the recommended RNI (10 µg/day) (SACN, 2016).

Table 4. 6: Dietary intake (amounts /d) and physical activity level (PAL) for 80 overweight and obese post-menopausal women stratified according to serum 25(OH)D concentration defined by (IOM), 2010.

	(<30 nmol/L)	**(≥30 nmol/L)**	**P value**
N	27 (33.7%)	53 (66.2%)	
Age (years)*	59.0 (57.0, 68.0)	61.0 (56.5, 67.5)	0.80
Weight (kg)*	77.0 (64.0, 93.5)	72.2 (67.4, 79.7)	0.24
Height (cm)	161.5 (7.0)	159.5 (5.9)	0.18
BMI (kg/m2)*	29.0 (26.0, 33.0)	28.8 (25.9, 31.3)	0.40
FM (kg)	32.4 (13.0)	30.6 (8.7)	0.46
PAL (METs/wk^2)*	2079.0 (198.0, 3318.0)	1782.0 (720.0, 4039.0)	0.57
Energy (Kcal/day)*	2358.1 (1856.2, 3215.6)	2562.0 (2049.5, 3357.1)	0.22
Carbohydrate (g)*	286.5 (228.7,394.9)	298.7 (230.0,402.5)	0.44
Calcium (mg)*	886.2 (809.8, 1149.4)	886.1 (728.2, 1216.0)	0.42
Fat (g)*	85.5 (66.6, 123.6)	96.3 (73.1,139.3)	0.26
Iron (mg)*	16.5 (13.6, 22.2)	17.8 (14.4, 21.3)	0.41
MUFA (g)*	20.8 (16.2, 27.6)	25.4 (18.5,35.5)	0.11
Sodium (mg)*	3355.8 (2767.7, 4451.6)	3876.9 (3050.7, 5061.7)	0.26
PUFA (g)*	11.3 (7.8, 17.5)	13.5 (10.0, 20.8)	0.11
Protein (g)*	92.6 (74.5, 118.9)	101.2 (77.7, 133.7)	0.25
SFA (g)*	23.6 (17.0, 36.9)	29.2 (19.4, 40.0)	0.16
Vitamin B12 (µg)*	3.7 (2.7, 5.4)	5.1 (4.1, 6.4)	**0.01**
Vitamin B6 (mg)*	2.7 (1.7, 3.4)	2.9 (2.1, 3.5)	0.22
Vitamin C (mg)*	201.7 (140.0, 267.0)	182.9 (129.4,257.8)	0.79
Vitamin D (µg)*	2.7 (1.9, 3.9)	3.6 (2.4, 4.8)	**0.02**
Vitamin E (mg)*	10.5 (7.7, 11.9)	10.8 (7.9, 15.3)	0.16
Zinc (mg)*	11.4 (8.7, 13.6)	11.9 (9.2, 15.1)	0.32
Nitrate Intake (mg/day)*	274.3 (131.7, 357.0)	200.3 (153.2, 331.0)	0.79

*Reported as median (25th, 75th percentiles). *t*-test for independent samples has been used for the analyses. BMI, Body Mass Index; FM, Fat Mass; PAL, Physical Activity Level; MUFA, Monounsaturated Fatty Acid; PUFA, Polyunsaturated Fatty Acid; SFA, Saturated Fatty Acid. Significant p values are shown in bold.

4.4.3 Blood pressure and assessment of vascular health

Mean blood pressure (BP) measurements and EF measurements (PWV and PORH) were comparable for the two groups of participants stratified by serum 25(OH)D concentration. For BP measurement, subjects with serum 25(OH)D concentration less than 30 nmol/L had higher

SBP and DBP but these values were not significantly different when compared with those in the higher serum 25(OH)D concentration (≥30 nmol/L) group **(Table 4.7)**.

For the assessment of PWV, the reference value for PWV is 12 m/s has been proposed in the 2007 European Society of Cardiology (ESC) and European Society of Hypertension (ESH) hypertension guidelines (Mancia *et al.*, 2007). The finding reveals that PWV is significantly higher in the <30 nmol/L serum 25(OH)D concentration ($p<0.05$). However, the mean values for both groups (9.2 (5.0, 11.7) for <30 nmol/L group and 8.5 (7.5, 9.8) in ≥30 nmol/L group) are less than the reference value, which can be considered as normal PWV. Pulse transit time (PTT), an index of arterial stiffness, was significantly higher in the lower serum 25(OH)D concentration group (<30 nmol/L) ($p<0.05$). PTT is a direct indicator of arterial pressure, since arterial stiffness increases as arterial pressure increases (Sharwood-Smith *et al.*, 2006).

There were no significant differences in PORH variables between the two groups stratified by serum 25(OH)D concentration. A reactive hyperaemia index (RHI) score of less than 1.67 is correlates with endothelial dysfunction (Schoenenberger *et al.*, 2012). In this study, the <30 nmol/L and ≥30 nmol/L groups have median RHI indices of 3.2 (2.5, 4.4) and 3.6 (2.3, 5.2), respectively (reported as median (25th, 75th percentiles)), which can be considered as normal RHI.

Table 4. 7: Physiological measurements of vascular health in 80 overweight and obese post-menopausal women stratified according to serum 25(OH)D concentration (IOM 2010)

	(<30 nmol/L)	**(≥30 nmol/L)**	**P value**
N	27 (33.75%)	53 (66.25%)	
SBP*	134.0 (123.0, 144.0)	127.0 (121.0, 143.5)	0.70
DBP	75.9 (9.1)	73.9 (9.7)	0.30
HR	65.7 (8.3	66.0 (10.7)	0.90
PORH variables			
• Resting level*	10.6 (8.1, 13.7)	10.0 (8.1, 13.0)	0.60
• ML*	77.0 (60.1, 96.2)	92.8 (67.5, 115.6)	0.12
• Area of occlusion*	1064.7 (750.7, 1878.9)	1160.7 (668.3, 1603.6)	0.45
• Area of hyperemia*	1584.4 (1137.6, 1992.3)	1696.0 (960.9, 2686.1)	0.57
• AH/AO*	1.41 (0.7, 2.3)	1.6 (0.9, 2.5)	0.29
• Time to recovery*	0.6 (0.5, 1.0)	0.8 (0.6, 1.0)	0.39
• Time to maximum*	11.7 (8.7, 12.8)	10.4 (7.02, 14.9)	0.97
• Time to half decay from maximum*	22.6 (18.9, 26.1)	21.8 (16.9, 25.3)	0.72
• RHI Index*	3.2 (2.5, 4.4)	3.6 (2.3, 5.2)	0.92
Phothophlethysmopghy			
• PTT_{40mmHg} (ms)*	71.0 (65.0, 81.0)	66.0 (55.0, 73.5)	**<0.05**
• PWV_{40mmHg} (m/s)*	9.2 (8.0, 11.7)	8.5 (7.5, 9.8)	**<0.05**

*Reported as median (25^{th}, 75^{th} percentiles). *t*-test for independent samples has been used for the analyses. SBP; Systolic Blood Pressure, DBP; Diastolic Blood Pressure, HR; Heart Rate, PORH; Post Occlusive Reactive Hyperemia, ML; Maximum Level, AH/AO; Area of Hyperaemia/ Area of Occlusion, RHI; Reactive Hyperemia Index, PTT; Pulse Transit Time, PWV; Pulse Wave Velocity. Significant p values are shown in bold.

4.4.4 Biomarkers of vitamin D and calcium status

Table 4.8 summarises biomarkers of vitamin D and calcium status. Except for calcium, the data are reported as medians (25th, 75th percentiles) as the data were not normally distributed. As was expected, mean serum 25(OH)D was significantly ($p<0.001$) greater – almost three times higher – in the higher vitamin D status group.

Table 4. 8: Biomarkers of vitamin D and calcium status in 80 overweight and obese post-menopausal women stratified according to vitamin D status

	(<30 nmol/L)	(≥30 nmol/L)	P value
N	27 (33.75%)	53 (66.25%)	
25(OH)D (nmol/L) *	21.0 (14.0, 27.0)	55.0 (42.5, 69.5)	< **0.05**
PTH (pmol/L) *	4.9 (4.2, 6.1)	4.5 (3.7, 6.0)	0.13
Calcium (mmol/L)	2.0 (0.13)	2.0 (1.8)	0.90

*Reported as median (25th, 75th percentiles). *t*-test for independent samples has been used for the analyses. If needed, data were log-transformed prior to analysis. Significant p values are shown in bold.

4.4.5 Sun exposure to measure the impact of dermal synthesis of serum 25(OH)D concentration

To investigate the possible impact of dermal synthesis of serum 25(OH)D concentration, participants completed a sun exposure questionnaire which assessed their habitual sunshine exposure during weekdays and at the weekend. Those participants who spent less than one hour in outdoors activities during the sunny months were defined as low- moderate sun exposure and those spending more than one hour as high sunshine exposure. Interestingly, the finding revealed that more than 50% of the participants in serum 25(OH)D concentration ≥ 30 nmol/L group are in the category of low-moderate sunshine exposure **Figure 4.6**.

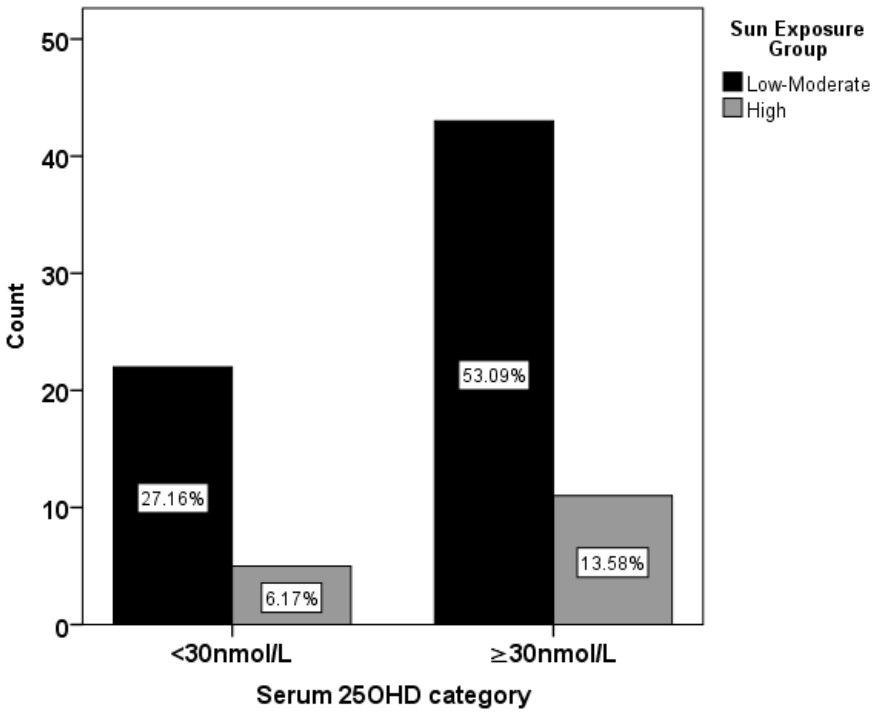


Figure 4. 6: Stratification of serum 25(OH)D concentration according to the sun exposure

4.4.6 Nitroso-compounds, biomarkers of EF and NO activity

Descriptive statistics for nitroso-compounds, biomarkers of EF and NO activity stratified according to the serum 25(OH)D concentration are presented in **Table 4.9**. While the low serum 25(OH)D concentration group (<30 nmol/L 25(OH)D) had apparently higher serum levels of ET-1 (p=0.61), urinary nitrate (p=0.83) and VEGF (p=0.99), these differences were not significant. However, ADMA concentrations were 38% higher in the group with <30 nmol/L serum 25(OH)D concentration and there was a non-significant trend of higher ADMA concentrations in the low vitamin D group (p=0.07). The current sample size provided a power of 36% to detect a significant difference between group and minimum total sample size of 160 participants (1:1 distribution between groups) would be needed to detect a significant difference in ADMA levels between low and normal serum 25(OH)D groups.

Table 4. 9: Descriptive of biomarkers of vascular health in 80 overweight and obese post-menopausal women stratified according to vitamin D status

	(<30 nmol/L)	**(≥30 nmol/L)**	**P value**
Plasma nitrate (µmol/L) *	27.3 (18.9, 38.1)	29.7 (22.9, 40.6)	0.67
Urinary nitrate (mmol/L) *	6.3 (3.6, 8.8)	5.4 (3.8, 10.19)	0.83
cGMP (pmol/mL)*	24.8 (18.5, 39.36)	25.8 (21.3, 35.1)	0.83
VEGF (pg/ml)*	110.9 (80.3, 137.7)	106.5 (71.4, 160.2)	0.99
ADMA (ng/mL)*	86.7 (54.7, 151.6)	54.3 (39.0, 128.2)	0.07
ET-1 (pg/ml)*	7.6 (4.5, 10.5)	6.9 (4.2, 11.2)	0.61
3NT (ng/mL)*	334.1 (85.4, 619.5)	382.5 (134.3, 894.8)	0.58

*Reported as median (25th, 75th percentiles). *t*-test for independent samples has been used for the analyses. If needed, data were log-transformed prior to analysis.
cGMP; cyclic guanosine monophosphate, VEGF; vascular endothelial growth factor, ADMA; asymmetric dimethylarginine, ET-1; endothelin-1, 3NT; nitrotyrosine

4.4.7 Correlation between serum 25(OH)D concentration and cardio-metabolic biomarkers

Table 4.10 reports correlations between serum 25(OH)D concentration and biomarkers of bone health, vitamin D and nitrate intake, blood pressure, vascular measurement and biomarkers of EF where significant correlations are highlighted in bold.

There was a positive correlation between PTH with DBP (r= 0.32, p<0.01), BMI (r= 0.34, p<0.01) and WC (r= 0.33, p<0.01) and a negative correlation with ET-1 (r=-0.22, p<0.05). Calcium correlated positively with cGMP (r=0.30, p<0.01), VEGF (r=0.26, p<0.01) and ADMA (r=0.23, p<0.05). On the other hand, SBP correlated positively with DBP (r=0.56, p<0.01), PWV (r=0.41, p<0.01) and 3NT (r= 0.32, p<0.01).

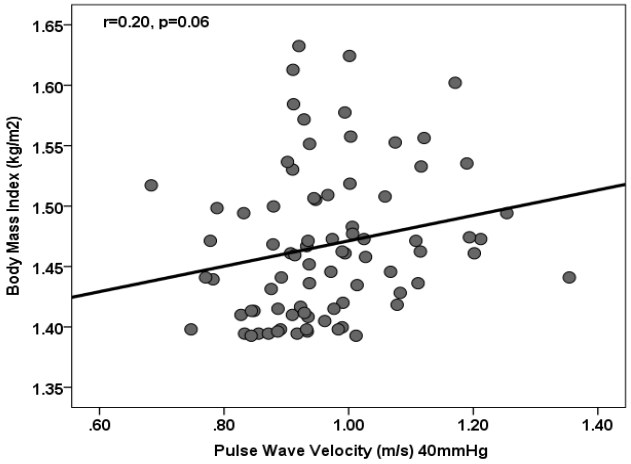
Diastolic blood pressure was positively correlated with PWV (r=0.48, P<0.01), BMI (r=0.32, p<0.01) and WC (r= 0.33, P<0.01). Plasma nitrate correlated positively with urinary nitrate (r=0.52, p<0.01), VEGF (r=0.22, p<0.05), 3NT (r=0.26, p<0.01) and age (r=0.22, p<0.05). Urinary nitrate correlated positively with VEGF (r=0.31, p<0.01) and VEGF correlated positively with ADMA (r=0.28, P<0.01) and 3NT (r=0.26, P<0.01).

Scatterplots were performed to observe the pattern of the correlations between the variables. **Figure 4.7 (A)** demonstrated the positive and almost significant (p= 0.06) relationship between BMI and PWV, while **Figure 4.7 (B)** shows the inverse association between BMI and plasma nitrate. On the other hand, **Figure 4.7 (C)** and **Figure 4.7 (D)** illustrated the positive correlations of age with ADMA, and of SBP with PWV, respectively.

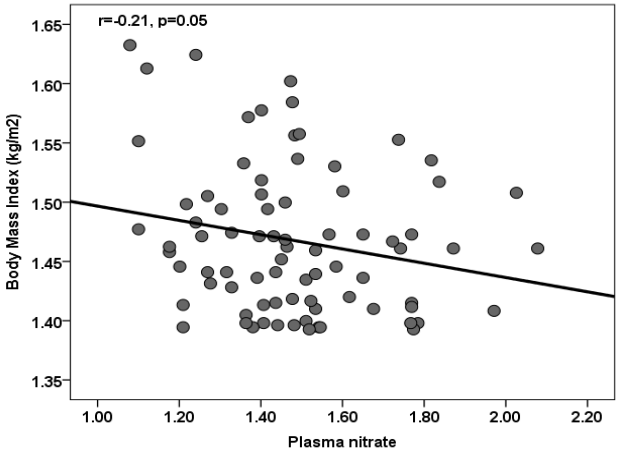
Table 4. 10 Correlation between 25(OH)D and anthropometric measurements, biomarkers of bone health, vitamin D and nitrate intake, blood pressure, vascular measurement and biomarkers of endothelial function

	25(OH) D	PTH	Calcium	Vit D Intake	NO_3 Intake	SBP	DBP	PORH Index	PWV	Plasma nitrate	Urinary nitrate	cGMP	ET-1	VEGF	ADMA	3NT	Age	BMI	WC
25(OH) D	1																		
PTH	r=-0.16 p=0.13	1																	
Calcium	r=0.01 p=0.96	r=0.13 p=0.23	1																
Vit D Intake	r=0.17 p=0.11	r=-0.03 p=0.76	r=-0.08 p=0.47	1															
NO_3 Intake	r=-0.04 p=0.69	r=-0.01 p=0.87	r=0.62 p=0.59	r=0.17 p=0.12	1														
SBP	r=0.02 p=0.98	r=0.15 p=0.18	r=0.13 p=0.24	r=-0.17 p=0.12	r=0.01 p=0.86	1													
DBP	r=-0.13 p=0.23	**r=0.32 p<0.00**	r=0.05 p=0.65	r=-0.21 p=0.05	r=0.09 p=0.42	**r=0.56 p<0.00**	1												
PORH Index	r=-0.01 p=0.95	r=0.01 p=0.87	r=-0.09 p=0.41	r=-0.07 p=0.52	r=0.03 p=0.74	r=-0.09 p=0.40	r=0.08 p=0.43	1											
PWV	r=0.18 p=0.11	r=0.13 p=0.23	r=0.00 p=0.98	r=0.05 p=0.63	r=0.07 p=0.53	**r=0.41 p<0.00**	**r=0.48 p<0.00**	r=-0.02 p=0.85	1										
Plasma Nitrate	r=0.09 p=0.41	r=0.00 p=0.99	r=0.14 p=0.18	r=0.14 p=0.20	r=0.01 p=0.88	r=0.00 p=0.96	r=0.00 p=0.97	r=0.05 p=0.61	r=0.06 p=0.58	1									
Urinary Nitrate	r=0.03 p=0.73	r=0.06 p=0.56	r=0.15 p=0.17	r=-0.06 p=0.56	r=0.03 p=0.78	r=-0.04 p=0.67	r=0.03 p=0.77	r=-0.08 p=0.44	r=0.03 p=0.74	**r=0.52 p<0.00**	1								
cGMP	r=0.01 p=0.92	r=-0.02 p=0.80	**r=0.30 p<0.00**	r=-0.06 p=0.55	r=-0.04 p=0.70	r=0.08 p=0.44	r=0.12 p=0.26	r=-0.16 p=0.15	r=-0.02 p=0.85	r=0.12 p=0.27	r=0.20 p=0.07	1							
ET-1	r=-0.18 p=0.11	**r=-0.22 p<0.05**	r=0.13 p=0.22	r=0.05 p=0.63	r=0.08 p=0.44	r=-0.04 p=0.67	r=-0.03 p=0.74	r=-0.03 p=0.74	r=-0.04 p=0.71	r=0.15 p=0.16	r=0.02 p=0.83	r=0.17 p=0.12	1						
VEGF	r=-0.03 p=0.77	r=0.04 p=0.66	**r=0.26 p<0.05**	r=-0.05 p=0.61	r=0.02 p=0.85	r=-0.01 p=0.88	r=-0.04 p=0.69	r=-0.19 p=0.78	r=-0.18 p=0.10	**r=0.22 p<0.05**	**r=0.31 p<0.00**	r=0.19 p=0.08	r=0.18 p=0.09	1					
ADMA	r=-0.11 p=0.30	r=0.10 p=0.37	**r=0.23 p<0.05**	r=0.05 p=0.61	r=0.05 p=0.62	r=0.04 p=0.70	r=-0.09 p=0.42	r=-0.04 p=0.67	r=-0.12 p=0.25	r=0.00 p=0.94	r=0.08 p=0.48	r=0.11 p=0.33	r=0.10 p=0.38	**r=0.28 p<0.05**	1				
3NT	r=0.12 p=0.27	r=0.02 p=0.79	r=0.16 p=0.14	r=-0.10 p=0.33	r=0.08 p=0.48	r=0.08 p=0.48	r=-0.03 p=0.75	r=-0.16 p=0.14	r=0.07 p=0.52	**r=0.26 p<0.05**	r=0.08 p=0.43	r=0.19 p=0.08	r=0.12 p=0.27	**r=0.26 p<0.05**	r=0.06 p=0.57	1			
Age	r=0.11 p=0.31	r=0.10 p=0.37	r=0.12 p=0.29	r=-0.06 p=0.58	r=-0.19 p=0.08	**r=0.32 p<0.00**	r=-0.01 p=0.29	r=-0.20 p=0.07	r=0.04 p=0.69	**r=0.22 p<0.05**	r=0.16 p=0.13	r=0.19 p=0.78	r=-0.01 p=0.88	r=0.17 p=0.12	r=0.21 p=0.05	r=0.11 p=0.30	1		
BMI	r=-0.11 p=0.29	**r=0.34 p<0.00**	r=0.06 p=0.55	r=-0.06 p=0.57	r=0.07 p=0.49	r=0.19 p=0.08	**r=0.32 p<0.00**	r=0.09 p=0.42	r=0.20 p=0.06	r=-0.21 p=0.05	r=-0.11 p=0.31	r=-0.04 p=0.67	r=-0.15 p=0.17	r=0.03 p=0.77	r=-0.02 p=0.80	r=-0.07 p=0.51	r=-0.11 p=0.29	1	
WC	r=-0.12 p=0.27	**r=0.33 p<0.00**	r=0.01 p=0.86	r=-0.02 p=0.81	r=0.11 p=0.31	r=0.17 p=0.13	**r=0.33 p<0.00**	r=0.20 p=0.07	r=0.15 p=0.17	r=-0.88 p=0.43	r=-0.07 p=0.51	r=0.01 p=0.89	r=-0.18 p=0.10	r=0.03 p=0.78	r=-0.02 p=0.85	r=-0.07 p=0.53	r=-0.04 p=0.66	**r=0.84 p<0.00**	1

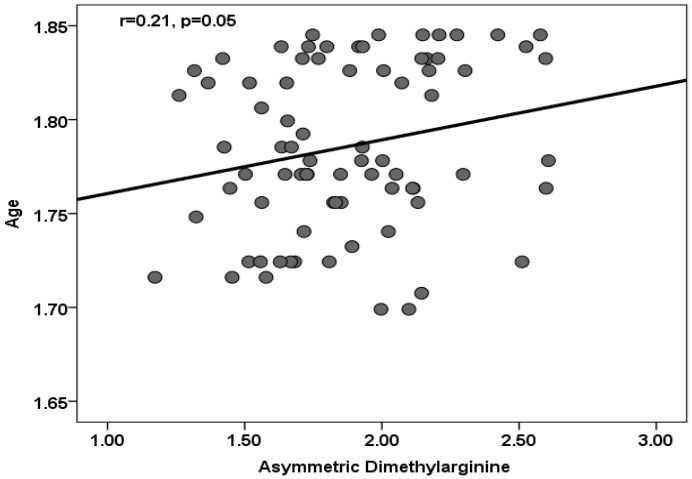
BP; blood pressure, BMI; body mass index, WC; waist circumference, FM; fat mass, PTH; parathyroid hormone, PTT; pulse transit time, PWV; pulse wave velocity, PORH; post occlusive reactive hyperemia, cGMP; cyclic guanosine monophosphate, ADMA; asymmetric dimethylarginine, VEGF; vascular endothelial growth factor, ET-1; endothelin-1, 3-NT; nitrotyrosine. Significant p values are shown in bold (Pearson's Correlation).



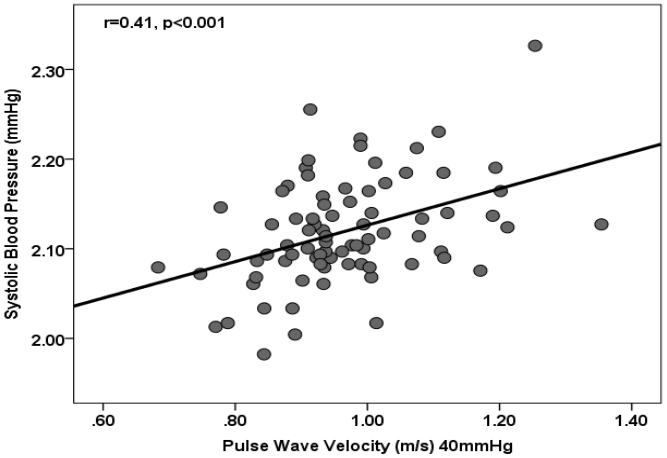
(A)



(B)



(C)



(D)

Figure 4. 7: Scatterplot of (A) BMI against PWV (B) BMI against plasma nitrate (C) Age against ADMA (D) SBP against PWV

4.4.8 Mean biomarkers of endothelial functions composite score

In this study, several biomarkers were assessed to measure endothelial functions including NO_3^-, cGMP, ADMA, 3NT, VEGF and ET-1. These biomarkers were not expressed in the same unit of measure. Therefore, a composite score, also known as *Z* score, was used to standardize the means of these biomarkers and to enable them to be combined into a single measure **(Figure 4.8)**. This approach has been described in detail in the method section. Whilst the mean EF composite score was lower in group with lower serum 25(OH)D concentration, the difference between groups was not significant (p = 0.31).

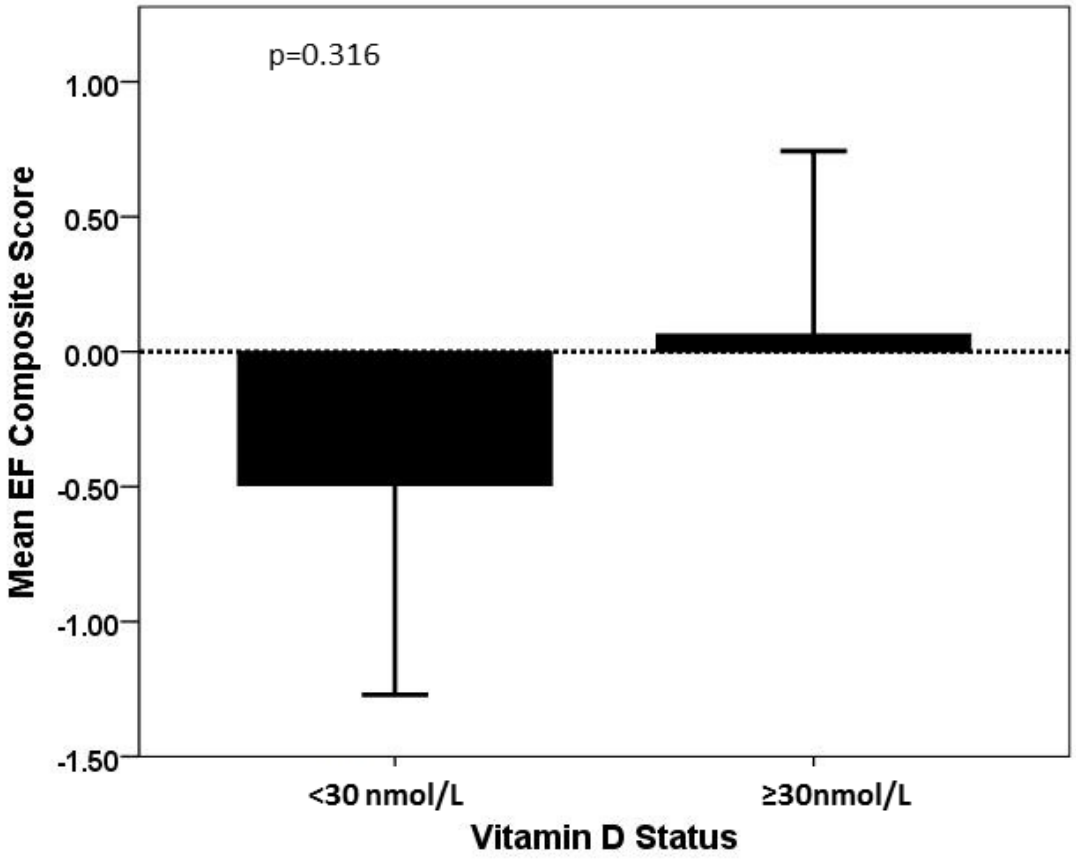


Figure 4. 8: The mean endothelial function composite score

4.4.9 Multiple linear regression analysis

Outcomes from the regression analysis model demonstrated that BMI was the most significant determinant of the arterial stiffness and that serum 25(OH)D concentration, cGMP, ADMA, age and PAL were not identified as independent predicting variables **(Table 4.11)**.

Table 4. 11: Results of multiple regression analysis for the dependent variable: Pulse Wave Velocity

	Pulse Wave Velocity (m/s)		
	B (SE)	**(95% CI)**	***P***
25(OH)D	0.07 (0.75)	-0.04 – 0.19	0.23
cGMP	0.01 (0.03)	-0.05 – 0.07	0.75
ADMA	-0.02 (0.01)	-0.06 – 0.01	0.23
Age	0.08 (0.15)	-0.22 – 0.39	0.59
BMI	0.21 (0.10)	-0.00 – 0.41	**0.04**
PAL	0.008 (0.02)	-0.07 – 0.06	0.76

Significant p values are shown in bold.
25(OH)D, 25-hydroxyvitamin D; cGMP, cyclic Guanosine Monophosphate; ADMA, Asymmetric Dimethylarginine; BMI, Body Mass Index; PAL, Physical Activity Level

4.5 Discussion

4.5.1 Main findings of the study

The serum 25(OH)D concentration in this sample of 80 overweight or obese post-menopausal women ranged from 12.0 – 125.0 nmol/L. Using a cut-off <30 nmol/L to indicate vitamin D 'deficiency' (Institute of Medicine Committee to Review Dietary Reference Intakes for Vitamin and Calcium, 2011), the study found that 27 participants (34%) were serum 25(OH)D concentration 'deficient'. This finding was compared with the biomarkers of vascular health in this group with those in the other 53 participants who had adequate serum 25(OH)D concentration. This revealed limited evidence for effects of serum 25(OH)D concentration on vascular function; for most biomarkers, including BP, there were no significant ($p>0.05$) differences between serum 25(OH)D concentration groups. However, PWV was significantly higher in the low serum 25(OH)D concentration (<30 nmol/L) group ($p<0.05$), which may

indicate greater arterial stiffness. In addition, there was a non-significant trend for higher ADMA concentrations in the low vitamin D group (p=0.07).

4.5.2 Interpretation of main findings

Sunshine exposure and seasonality

This study was conducted among women who live around Newcastle upon Tyne, Tyne and Wear. Situated in Northern England, with latitude 54-55ºN, the sunlight is too weak during the late autumn, winter and early spring (October to March) to produce cutaneous biosynthesis of vitamin D. A wide survey in the United Kingdom revealed that more than 50% of the adult population has insufficient circulating concentrations of vitamin D, and that the prevalence of insufficiency increased by 16% during winter and spring. The survey also demonstrated a gradient of prevalence across the UK, with highest rates in Scotland, northern England, and Northern Ireland (Hypponen and Power, 2007).

Season plays an important role in determining serum 25(OH)D concentration because it determines whether sun exposure results in endogenous cutaneous synthesis of vitamin D as a result of ultraviolet (UV)B radiation (Kimlin, 2008). In general, populations living >30ºN and >30ºS receive no UVB during the winter time, therefore cutaneous synthesis of vitamin D is impossible in this season (Schoenmakers *et al.*, 2016). This cross sectional study found that majority of the participants (65%) who are deficient for serum 25(OH)D were recruited during winter and spring. It is well acknowledged that the vitamin D supply during the absence of sun exposure is mainly through diet and supplementation.

The present study collected some information on factors which may influence sun exposure and, therefore endogenous vitamin D synthesis. Regarding the type of clothing worn, the majority of participants (95%) reported that they usually wear short sleeves and long trousers/skirt when there are outdoors during the sunny months. However, 70% of participants reported that they use sunscreen when being outdoors. As majority of the participants are in retirement age (mean age 61 years old), almost all of the participants spend most of their time indoors or mixture. Given that there is insufficient solar UVB to generate vitamin D in the skin at latitudes >50ºN, and that they spent limited time spent outdoors, the participants would not synthesis vitamin D especially during the winter/early spring months.

Anthropometric, physical activity and dietary intake

Our study demonstrated that there is no significant difference between the two serum 25(OH)D concentration groups with respect to anthropometry, physical activity and most aspects of dietary intake. However, from the food frequency questionnaire, we found that the lower serum 25(OH)D concentration group has significantly lower dietary intakes of vitamin B12 and vitamin D. There is no direct link between these two vitamins, but it might represents a common denominator as both vitamins needs a healthy intestine for the absorption (Mishra *et al.*, 2015). Vitamin D and vitamin B12 are absorbed in ileum. Both of these vitamins need assistance in order to be absorb in the intestine. Vitamin B12 combines with a protein made by the stomach called intrinsic factor and vitamin D needs VDR in the intestine. In this study, both serum 25(OH)D concentration groups have vitamin B12 intake more than the recommended RNI intake (RNI for vitamin B12 is 1.5 µg/day) (British Nutrition Foundation, 2016).

Dietary sources of vitamin D are limited and were mainly obtained from natural sources such as fatty fish and eggs. Some sources of vitamin D can be found in fortified food such as milk and dairy product, breakfast cereals and fat spread (Spiro and Buttriss, 2014). Natural sources of vitamin B12 were mainly from meat, fish, dairy products, poultry, eggs and fortified breakfast cereals (O'Leary and Samman, 2010). Therefore, it can be postulated that the sources of vitamin D and vitamin B12 in dietary intake of the participants were mainly from eggs, fish and breakfast cereals.

Biomarkers of bone health

Participants in this study had normal concentrations of PTH and calcium. PTH and vitamin D share a reciprocal relationship that links the status of calcium level in the body, where serum 25(OH)D concentration plays a main role in regulating calcium and PTH level. Normally, low blood calcium concentration triggers PTH release, which raises the circulating concentration of calcium. However, serum 25(OH)D concentration is essential for the absorption of calcium; vitamin D binds to VDR in enterocytes and stimulates active calcium transport from the intestine to the circulation which increases the concentration of calcium in blood (Lips, 2012). In this study, there was an inverse (negative) correlation between serum 25(OH)D concentration and PTH but this was not significant.

Physical activity

Physical activity can be an important determinant of serum 25(OH)D concentration as it may act as a surrogate for sunshine exposure during the outdoor physical activity (Wanner *et al.*, 2015). However, in this study, there was no significant difference in PAL between the two vitamin D groups. A self-report PA questionnaire (IPAQ-SF) (which assess the frequency and type of activities performed along with duration – number of minutes per week) was used to estimate the level of PA among the participants. Designed to be used in population-level surveillance of PA among adults, this questionnaire was structured to provide separate scores for three PA domains (walking, moderate-intensity and vigorous intensity activity). In this study, there were no significant differences between the two serum 25(OH)D concentration group for the three sets of domains.

Endothelial function measurements

There are various methods of assessing EF in humans depending on the resources, equipment available and technical skill (discussed in Chapter 3). In this study, PWV and PORH were used for the assessment of EF.

PWV was used to measure the arterial stiffness, and is inversely correlated with EF. Arterial stiffness is known as an early marker of systemic atherosclerosis. In this study, those who have lower serum 25(OH)D concentration were hypothesized to have higher PWV index, which reflect the higher arterial stiffness.

This study demonstrated that the lower serum 25(OH)D concentration group has significantly higher mean PWV. A similar finding was reported by (Giallauria *et al.*, 2012) who found a significant inverse association between PWV and serum 25(OH)D concentration (adjusted r^2 =0.27; β=-0.43; P=0.001). A recent cross sectional study among Korean adults found that arterial stiffness was higher in those with lower serum 25(OH)D concentration (Lee and Suh, 2017). However, several previous cross sectional studies reported no significant association between serum concentration of 25(OH)D and PWV (Lim *et al.*, 2012; Deleskog *et al.*, 2013).

PORH, measured as reactive hyperaemia index (RHI) was the second method of measuring EF in this study. Those who have lower serum 25(OH)D concentration were hypothesized to have lower RHI. Impaired endothelial function is defined as RHI < 1.67 (Syvänen *et al.*, 2011). However, the results demonstrated that the RHI for both serum 25(OH)D concentration groups

was more than 1.67 (median 3.2 for < 30nmol/L serum 25(OH)D concentration and median 3.6 for ≥ 30nmol/L serum 25(OH)D concentration). The finding also showed that there is no significant difference for the mean of RHI between the two serum 25(OH)D concentration groups. This is in line with a recent study done among premenopausal African American women which reported that serum 25(OH)D concentration did no associated with mean of RHI ($p>0.05$) (Moore and Ruppe, 2017). Contrary to this result, a study done by (Ertek *et al.*, 2012) revealed that the mean RHI was significantly lower in serum 25(OH)D concentration deficient subjects than in those with normal serum 25(OH)D concentration group.

Biomarkers of EF

There are various biomarkers of EF including cGMP, ADMA, VEGF, ET-1, vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1), endothelial leucocyte adhesion molecule (ELAM-1) (Dessein *et al.*, 2005), interleukin 6 (IL-6), 4-hydroxynonenal (4-HNE), fibroblast growth factor-23 (FGF-23), e-selectin and p-selectin. In this study, the nitroso-compound biomarkers group (cGMP, ADMA, VEGF and ET-1) were measured as these four biomarkers are closely related to NO production. The role of these biomarkers has been discussed in Chapter 3.

The results for nitroso-compounds demonstrated that those who in higher serum 25(OH)D concentration group had higher plasma nitrate and those who had lower serum 25(OH)D concentration had higher urinary nitrate. This study also showed that participants in the low vitamin D status group (<30 nmol/L) have higher ADMA concentration - this difference was almost statistically significant ($p=0.07$). As been described in Chapter 3, ADMA is closely related to endothelial dysfunction. Elevated levels of ADMA inhibit NO synthesis which leads to impairment of EF, resulting in atherosclerosis (Yilmaz *et al.*, 2008).

Increased arterial stiffness and endothelial dysfunction are strong predictors for future development of hypertension (Quyyumi and Patel, 2010). The majority of large cross-sectional studies have demonstrated an inverse relationship between serum 25(OH)D concentration and blood pressure, and the risk of future hypertension seems to be greater in those with vitamin D deficiency (Forman *et al.*, 2007; Pilz *et al.*, 2009). In this study, we observed a significant correlation between PWV with SBP and DBP ($p<0.001$). Similarly, a study done among normotensive and hypertensive Malay men demonstrated that PWV was significantly higher in hypertensive compared with the normotensives (blood pressure 169/100 mm Hg +/- 14/7

vs. 120/80 mm Hg +/- 10/4, p < 0.001; PWV 11.69 m/s +/- 1.12 vs. 8.83 m/s +/- 1.35, p < 0.001) (Ngim *et al.*, 1999).

There was a trend of positive correlation between BMI and PWV (p=0.06) and a significant correlation between BMI and DBP (p<0.001). Obesity is an important risk factor for hypertension, with obese people having higher rates of hypertension than normal weight individuals (Mertens and van Gaal, 2000; Lakoski *et al.*, 2011).

A mechanism by which obesity might contribute to arterial stiffness has been proposed. In obese people, macrophages are accumulating in adipose tissue (Yudkin *et al.*, 1999; Weisberg *et al.*, 2003; Boutens and Stienstra, 2016). This condition will leads to the chronic state vascular and systemic inflammation and leads to impaired endothelial function in obese people (Cancello *et al.*, 2006; Strasser *et al.*, 2015). It is also well established that higher BMI is associated with higher SBP and DBP due to greater cardiac output, increased blood flow, vasodilation and vascular resistance with increased weight (Felber and Golay, 2002; Dua *et al.*, 2014).

4.5.3 Public health implications of study

This finding revealed that the average daily dietary intake of vitamin D among the study participants was 3.82 µg/day, which is much lower than the recommended daily dietary intake of vitamin D (10 µg/day) (Ashwell *et al.*, 2010; SACN, 2016). This is in line with results from UK National Diet and Nutrition Survey which reported that the prevalence of inadequate dietary intakes of vitamin D among older adults in UK is high and that dietary vitamin D intake is approximately 2-3 µg/day (Smithers *et al.*, 2000). Living in the northern hemisphere at latitudes greater than 40ºN, with restricted sunlight to produce cutaneous vitamin D synthesis, the UK population is at risk of vitamin D deficiency if dietary intake is low. Apart from supplementation, vitamin D can be obtained by consuming dietary vitamin D such as fatty fish, eggs, dairy products, fortified milk and breakfast cereals (Spiro and Buttriss, 2014).

Therefore, there is a need to address these problems at national and regional level with aim of early identification and prevention of vitamin D deficiencies. Appropriate community based intervention program should be reinforced to increase the awareness of the community of the importance of vitamin D.

4.5.4 Strengths and limitations of the study

This study has been conducted among 80 overweight and obese postmenopausal women in Newcastle, United Kingdom. This population group was chosen for the study because such women are at increased risk of vitamin D deficiency (Snijder *et al.*, 2005) and because their vascular function will be more compromised than in younger and leaner women (Celermajer *et al.*, 1994). In this sample, 1/3 of the participants had serum 25(OH)D concentration less than 30 nmol/L (the cut-off for the deficiency as defined by the IOM (Institute of Medicine Committee to Review Dietary Reference Intakes for Vitamin and Calcium, 2011).

A high variability in BP measurements were found among the participants. Although the mean BP was normal for both groups of participants stratified by serum 25(OH)D concentration, the inter-individual variation shows more than 1/3 of the participants were categorised as having high blood pressure (SBP >140 mmHg and DBP > 90 mmHg). The heterogeneity in serum 25(OH)D concentration and biomarkers of vascular function also provided a foundation for further investigation whether serum 25(OH)D concentration was associated with better EF. Furthermore, all observations in this study were done by the same researcher, which may reduce the likelihood of confounding by inter-researcher differences in measurements.

This study has a few limitations. The study was restricted to postmenopausal women aged between 50 to 70 years with BMI more than 24.9 kg/m^2. Subjects were not randomly selected, but volunteered to participate, which may contribute to bias. Since during ageing, men showed progression of endothelial dysfunction earlier than women (Celermajer *et al.*, 1994), older men might be more sensitive to the effects of vitamin D compared to older women. Furthermore, this study used a single measurement of serum 25(OH)D concentration as an indicator of vitamin D status and this may not be sufficient to reflect accurately long-term vitamin D status. This study also did not consider the seasonal variation of serum 25(OH)D concentrations, which is necessary to develop assessment of vitamin D deficiency and vitamin D supplementation strategies in the future study.

4.5.5 Conclusion

In this study, we found that there is no significant association between serum 25(OH)D concentration with biomarkers of EF, blood pressure, age and BMI. However, our study demonstrates that there is a significant difference in mean PWV between lower and higher

serum 25(OH)D concentration groups. Those participants with higher serum 25(OH)D concentration had lower mean value of PWV compared to lower serum 25(OH)D concentration ($p<0.05$). However, this study was restricted to women aged 50 to 70 years and it will be important to extend such studies to older women and to men before drawing definitive conclusions about the relationship between serum 25(OH)D concentration and vascular health. In addition, longer term (more than 12 months) vitamin D intervention studies in those with compromised vitamin D status will be necessary to provide causal evidence for a role for vitamin D in maintenance endothelial function and for cardio-metabolic health.

Chapter 5 – Ageing, Vitamin D and NO Production

5.1 Introduction

The normal function of the cardiovascular system is closely dependent on the integrity of the endothelium, a monolayer of cells lining in the lumen of blood vessels (Teixeira *et al.*, 2014). The loss of integrity of the endothelial layer and consequent alteration of the function of endothelial cells may trigger critical mechanisms (e.g. increased monocyte and platelet adhesiveness, loss of laminar flow and vascular elasticity) involved in the pathogenesis of cardiovascular diseases (CVD) (Rajendran *et al.*, 2013). These include coronary heart, cerebrovascular and peripheral vascular diseases, which are largely attributed to the atherosclerotic lesions (Widmer and Lerman, 2014).

Ageing is one of the most important CVD risk factor, due to structural alterations and loss of function in the vasculature during the ageing process (Seals *et al.*, 2011). These vascular changes affect both smooth muscle cells and intima layers. The mechanisms underlying vascular ageing are complex and involve multiple pathways and factors such as reduced peripheral artery endothelium-dependent dilation, arterial stiffness, impaired angiogenesis, increased vascular oxidative stress and increased superoxide production (Seals *et al.*, 2011). Vascular function depends on availability of nitric oxide (NO), which is synthesised mainly by the activity of endothelial nitric oxide synthase (eNOS) on the substrate arginine (Förstermann & Sessa, 2012). NO helps keep blood vessels dilated, controls blood pressure, and has numerous other vasoprotective and anti-atherosclerotic effects (Förstermann & Sessa, 2012). Ageing is associated with impaired endothelial function which may be due to decreased synthesis of NO. Several studies have evaluated the association between ageing and physiological (e.g. flow mediated dilation) or biochemical (i.e., nitrate, nitrite) indirect markers of NO synthesis and have reported reduced synthesis of NO with ageing (Toprakçı *et al.*, 2000). However, to date, no study in humans has quantified the relationship between ageing and whole-body NO production measured by stable isotopic tracers. There is evidence that concentrations of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS increase with ageing, which may contribute to lower whole body NO production and deterioration of endothelial function (Sverdlov et al., 2014). Siervo *et al* 2011 have reported a significant association between ADMA concentration and whole body NO production in obese

individuals (Siervo *et al.*, 2011b) but no study has investigated whether there is an association with ageing.

Vitamin D may play a key role in modifying the risk for adverse cardio-metabolic outcomes, particularly metabolic syndrome (MetS), type 2 diabetes mellitus, and systemic hypertension (Lavie et al, 2011). Whilst the mechanisms for these associations are not clear, these may relate to the role of vitamin D in modulating NO production (Andrukhova *et al.*, 2014) . Vitamin D is involved in the regulation of endothelial cell-dependent vasodilation, an effect that may be mediated by the VDR, which cause the phosphorylation of p38, Akt and ERK (which play important roles in cell proliferation and survival). Activation of this MAPK signalling pathway leads to eNOS activation and, increased NO synthesis, which in turn, may improve the modulation of vasculature (Tousoulis *et al.*, 2012; Dalan *et al.*, 2014).

Ageing may impact on 25(OH)D status. The formation of the active form of vitamin D ((1,25[OH]$_2$D; calcitriol) may be reduced as a result of age-related decline of renal function by, reducing the activity of the enzyme 1-α hydroxylase that converts 25(OH)D into 1,25(OH)$_2$D in the kidney (Gallagher, 2013). In addition, skin production of 25(OH)D may decrease in older people due to lower 7-DHC activity in the epidermis (MacLaughlin and Holick, 1985; Kinyamu *et al.*, 1997a; Kinyamu *et al.*, 1997b; Gallagher, 2013).

In this study, the association of whole-body NO production measured using a non-invasive stable isotopic method with age, gender and serum 25(OH)D concentration was investigated for the first time. Finally, the associations between serum 25(OH)D concentration and whole-body NO production, PWV and plasma concentrations of nitrite and ADMA were explored.

5.2 Aims and Objectives

5.2.1 Hypothesis

Ageing is one of the most important risk factors for endothelial dysfunction and impaired cardiovascular health which may be partially explained by an age-related decline in NO production. This study aims to test the hypothesis that whole-body NO production is reduced in older compared to younger individuals and test whether these differences could be explained by differences in circulating 25(OH)D concentrations. Previous research using stable isotopic methods (Forte et al., 1997) has indicated a greater production of NO in women compared to

men and this aims to confirm these previous findings and whether this gender-specific association with NO production may be modified by age (young vs old).

Primary Objectives:
To investigate the association between age and whole body NO synthesis.

Secondary Objectives:
To determine the association between whole body NO synthesis with:

- Circulating 25(OH)D concentrations
- Gender
- Resting-clinic and home blood pressure measurements
- Pulse wave velocity
- Plasma ADMA, nitrate and nitrite concentrations

5.3 Methods

5.3.1 Study design

This was a cross sectional study involving both younger (20-49 years) and older (50-75 years) with equal numbers (10 males and 10 females) within each age group. A telephone screening interview was conducted to check for participants' eligibility according to the inclusion/exclusion criteria (see below). Potentially eligible individuals were invited to Newcastle University's NU Food Research Facility to confirm eligibility, to obtain verbal and written informed consent and to start the study that took place between June 2017 and July 2017.

5.3.2 Participants

The study aimed to recruit volunteers from the general public living in Newcastle. Forty healthy, non-smoking, normal weight and overweight (BMI: 20.0 – 29.9kg/m^2) males and females were recruited. Participants were excluded if they had any of the following criteria: currently participating in other clinical study; vegetarianism (likely to have very high nitrate intake); high physical activity level (may have BMI in obese range but low fat mass), weight change more than 3.0 kg in the last two months (important influence on systemic metabolism and vascular function); active cancer and any diagnosis of malignant cancer in the last five years (likely to have systemic effects on study outcomes); chronic and acute metabolic and inflammatory conditions interfering with the study outcomes; previous diagnosis of type-1 or

type-2 diabetes treated with insulin and oral hypoglycaemic agents (modification of regulation of intermediate metabolism; weight loss medications (sibutramine, orlistat, rimonabant), history of bariatric surgery; drug use including: oral corticosteroids, anticoagulants, nitrate derived agents, anti-cholinergic; subjects on hormonal therapies (oestrogens, thyroxine, progesterone, oral hypoglycaemic agents), anti-hypertensive (diuretics, beta-blockers, calcium antagonists, ace-inhibitors and angiotensin receptors inhibitors), statins and any other anti-dyslipidaemic agent and psychiatric drugs (antidepressants, sedatives, antipsychotics) - individuals will be excluded if dose for the drugs has been started/changed in the previous six months; alcohol intake >21 units/week for men and >14 units/week for women.

5.3.3 Sample size

Power calculations were based on differences in whole-body NO production seen in previous studies conducted by Siervo and colleagues using the same stable isotope method (Siervo *et al.*, 2011b). Siervo estimated a normal production of NO as 0.63±0.25µmol*hr^{-1}*kg^{-1}. If we assign this reference value to the young healthy group, a 20% difference in whole-body NO production (with the same variability) between age groups will be detected with a total sample size of 40 subjects (20 subjects per age group, independent t-test) with an α-error probability of 0.05 and a 1-β error probability of 0.80. G Power 3.1 for Windows was used for the calculation of sample size.

5.3.4 Ethical approval

Prior to recruiting any participants, ethical approval was sought and gained from the Faculty of Medical Sciences, Newcastle University (1285/15004/2017) **(Appendix 5.1)**.

5.3.5 Recruitment

Telephone interview

Participants were provided with all the pertinent information regarding the study during the telephone interview. The participants were also asked for information about contact details, anthropometric measurement, health status, medication they consumed and smoking and drinking status. If they appeared to be eligible and agreed to participate in the study, an appointment was set up based on the availability of the participant to attend the study.

Preparatory protocol (prior to study visit)

An invitation email was sent to the potential participants confirming the date, time and venue of the study visit. The participant information sheet also was attached to the email. Participants

were also informed to refrain from eating and drinking caffeinated drinks at least four hours before the visit. They were also asked to minimise nitrate intake a day before the study visit and a detailed list of allowed and not allowed food items were emailed to the participants **(Appendix 5.2)**.

5.3.6 Clinical assessment

The study was conducted at the NU Food research facilities between 8.30 am and 12.30 pm. After confirming the participant's eligibility, samples of blood, urine and saliva were collected. Measurements of body weight and body composition (Bioelectrical Impedance Analysis) were performed. Resting blood pressure was measured in triplicate using an automated device. Vascular measurement (PWV) was performed to assess arterial stiffness. Participants were provided with eight pre-labelled 2 ml tubes for the collection of saliva samples at 5 pm (pre-dose), 7.30 pm, 8.15 pm, 9.00 pm, 10.00 pm and the next morning at 7.00 am, 8.00 am and 9.00 am. A dose of labelled sodium nitrate ($Na^{15}NO_3$, 99% Sigma Aldrich, UK) dissolved in 100 ml of nitrate free water (Buxton water) was drunk by each participant immediately after the collection of the pre-dose saliva sample (at 5.00 pm).

The participants were provided with a folder containing the questionnaires for the assessment of dietary intake (MEDE Food Frequency Questionnaire and nitrate intake questionnaire), lifestyle (IPAQ questionnaire) and saliva sample collection procedures **(Appendix 5.3)**. The participants were instructed to collect saliva samples and to measure BP at home. A small meal (two packs of muesli bars) and nitrate free water were provided to be consumed by the participants during the study. After the collection of the last saliva sample (at 9.00 am), the study was completed **(Figure 5.1)**. The participants were required to return the saliva samples, blood pressure device, study forms and questionnaires on the next day at the research centre. For those who having difficulties to go to the research centre, the investigators went to collect the materials at the participants' home.

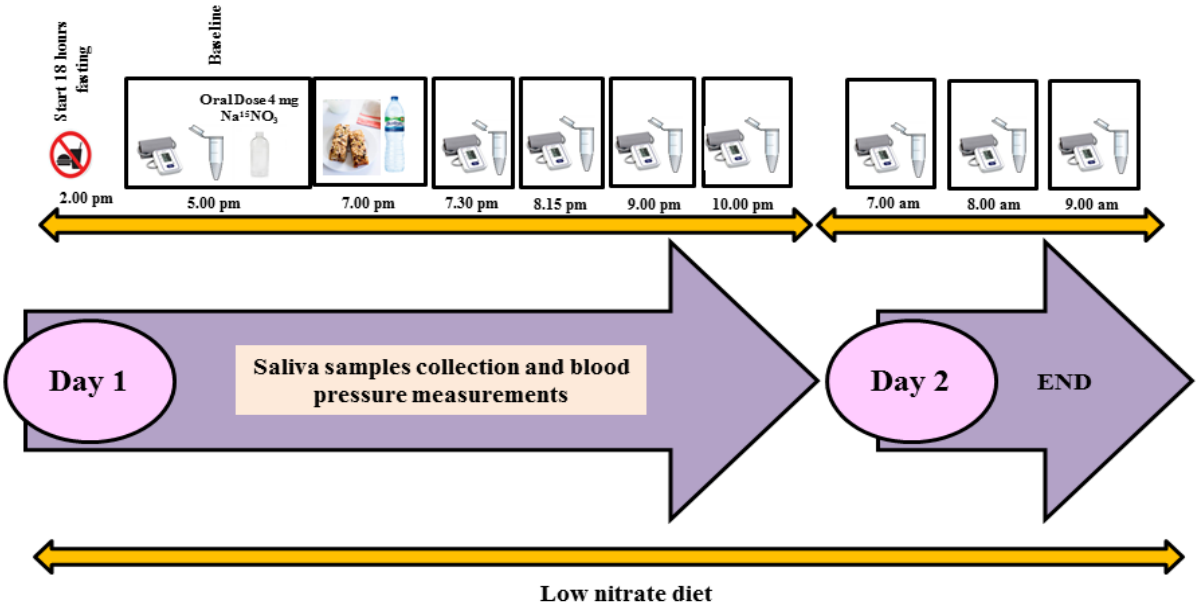


Figure 5. 1: Overview of the protocol

5.3.7 Measurement Protocols

Questionnaires

Participants were asked to complete a set of questionnaires on general health and lifestyle. In addition, each participant's diet was assessed using a validated food frequency questionnaire and a nitrate intake questionnaire, and each participant was asked to complete a sun exposure questionnaire.

Body composition

Measurements were performed during the visit. Height and weight were measured with a standard stadiometer and weight scale, with participants wearing light clothing and barefooted. Weight was measured in kilograms to the nearest 100 g. Weight and height measurements were used to calculate BMI according to the following equation:

$$BMI = \frac{\text{weight (kg)}}{\text{height (m)}^2}$$

Waist circumference was measured with a standard non-stretchable tape, in the standing position, at the minimum circumference between iliac crest and the rib cage. Body composition was measured using a leg-to-leg bioelectrical impedance device (TANITA 300 MA).

Resting blood pressure and pulse wave velocity

Clinic: Blood pressure measurements were performed in triplicate at one minute intervals using an automated blood pressure monitor (model: Omron M3 [HEM-7200-E8(V)]). Before the measurement, participants were invited to rest in a sitting position for at least 15 minutes. An appropriate cuff size was utilised for the measurements. The average of the three measurements was used in the analysis.

Home: Participants were provided with the same automated BP monitor to measure their resting BP at home immediately after the collection of the saliva samples as part of the ONT method (see below). In total, eight BP measurements were recorded by the participants **(Appendix 5.4)**. The average of the eight measurements was calculated for both SBP and DBP ad used in the analysis.

Pulse wave velocity: Digital photoplethysmography (PTT) was used and details of the procedures are described in Chapter 4. Briefly, this measurement involved the attachment of five electrodes using small adhesive gel pads to the ear lobe, fingertip, chest and abdominal area. A series of two separate measurements were performed with different external cuff pressures (0 and 40 mmHg) applied to the whole right arm through the long cuff, with a minute rest interval between each measurement. The duration of this measurement was approximately 15 minutes.

Whole-body NO production

A non-invasive method was used to assess whole-body NO production (ONT Method) (Siervo *et al.*, 2011a). At the beginning of the ONT test, participants were asked to consume a meal with low nitrate content and the information was provided to them. Low nitrate bottled mineral water (0.1 mg/l) was provided to the participants. Subjects were received an oral dose of sodium nitrate (4 mg) in 100 ml distilled water (4 mg $Na^{15}NO_3$) and the isotopic decay of the tracer in saliva samples was used to derive estimates of NO production. Saliva samples were collected at predefined times over a period of 16 to 18 hours. Each participant was given a form for the saliva collection time and to write down the blood pressure measurements.

The principle of the method is based on an isotopic dilution model. The oral dose of nitrate is rapidly assimilated into plasma (complete absorption in under two hours), which, on the timescale of the disposal kinetics, can be regarded as a bolus dose. Linear interpolation was used to estimate the 2-h changes in concentrations and tracer-to-tracee ratio (TTR) during the measurement period. The isotopic decay in saliva of an oral dose of labelled nitrate was

described by an exponential function for a single compartment. Data was described using a semi-logarithmic plot and the slope and intercept of the regression line was used to derive the rate of NO synthesis.

All saliva samples were derivatised by nucleophilic substitution of mesitylene with trifluoroacetic acid anhydride (TFAA) as the catalyst to give a single product, nitromesitylene (1, 3, 5-trimethyl nitrobenzene). Gas Chromatography Mass Spectrometry (GCMS) was used to determine the level of enrichment. The full description of the method was described in Chapter 3. All analyses were performed in duplicate in selected ion monitoring (SIM) of the molecular ion (MO) at m/z 165 and the M+1 ion at m/z 166 representing the unlabelled and labelled nitro-mesitylene respectively. See also Chapter 3 for an example of typical unlabelled and labelled chromatograms and corresponding spectra.

NO_3^- and NO_2^- analyses

Plasma and urinary concentrations of NO_3^- and NO_2^- were analysed using a Sievers gas-phase chemiluminescence NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The Sievers NOA consists of two components, glass purge vessel and NO chemiluminescence analyser. The purge vessel contains either vanadium chloride or tri-iodide (I3) solutions. These solutions react with NO_3^- and NO_2^- (respectively) in the injected samples to produce NO. NO released from the above reactions are quantified by the chemiluminescence analyser. The concentrations of NO_3^- and NO_2^- were determined by plotting signal area (mV) against a calibration plot of a known concentration NO_3^- and NO_2^- standards. To analyse data from the injected samples, the Liquid Program (version 3.21) was used.

Plasma NO_3^- and NO_2^- were processed to remove the protein from the plasma sample before the analysis. The plasma samples collected in lithium heparin vacutainer were used for the analysis. Details for the deproteination procedures were described in Chapter 3. Urine samples for the measurement of NO_2^- required a 100-fold dilution (10 µl of urine samples + 990 µl of Milli-Q® water). A more detailed description of the protocol is provided in Chapter 3 and examples of calibration curves and sample analysis are provided in the **Appendix 5.5**.

Venepuncture/blood samples

Blood samples were collected after four hour fasting. Plasma samples were utilised to measure NO_3^-, NO_2^- and biomarkers of endothelial dysfunction. Serum plasma was used to measure 25(OH)D concentration. Blood samples were collected using the following vacutainer tube:

Lithium heparin (5.0 ml), EDTA (5.0 ml), plain tube (5.0 ml). The samples were immediately centrifuged within five minutes after collection to preserve NO_2^-.

Asymmetric Dimetylarginine (ADMA)

The measurement of ADMA in this PhD project was done using a commercial kit (CUSABIO ELISA kit CSB-E09298h) in a 96-well format. One hundred µl of plasma samples from EDTA tube was added per well. Details of the ADMA analysis procedures can be found in Chapter 3.

Vitamin D concentration analysis

Vitamin D concentrations analysis was done using commercial kit (25-Hydroxy Vitamin D^s Enzyme Immunoassay (EIA) from Immunodiagnosticsystems (IDS) AC-57SF1) in a 96-well format. Details of the 25(OH)D analysis procedures can be found in **Appendix 5.6**.

Principle of assay

The quantitative 25(OH)D ELISA kit uses a microtiter plate format. 25 µL of each calibrator, controls and serum samples were diluted with biotin labelled 25(OH)D. The diluted samples were incubated in microtiter wells with a fixed number of highly specific sheep 25(OH)D antibody at room temperature before aspiration and washing. Enzyme (horseradish peroxidase) labelled avidin was added and binds selectively to complexed biotin and the chromogenic substrate (TMB) was added to developed the colour. The stop solution was added to stop the reaction mixtures, and the fluorescent signal was monitored at 450 nm. The colour intensity developed is inversely proportional to the concentration of 25(OH)D. Therefore, the higher the sample 25(OH)D concentration, the weaker is the intensity of the signal.

Saliva samples collection

Saliva samples were obtained for the measurement of nitrate isotopic enrichments during the ONT method. Samples were collected by chewing a cotton ball for about two to three minutes and then using the 20 ml syringe to squeeze the saliva in the cotton ball into 1.5 ml Eppendorf tube. The tubes were containing 5 µL of 1M sodium hydroxide solution to prevent nitrate degradation. Samples were stored at -20ºC until further analyses.

Urine Collection

A spot urine sample was collected at the visit in urine plastic container. First void urine was discarded. Urine was then aliquoted in two Eppendorf tubes and stored at -20ºC for future analyses. Urine sample was used for the assessment of NO_3^- concentrations using chemiluminescence.

Dietary assessment

A food frequency questionnaire for the assessment of overall dietary and NO_3^- intake was administered to evaluate typical energy and nutrient intake of each participant. The intakes of energy and nutrients were calculated using the MEDE FFQ Database.

5.3.8 Sample storage

Blood samples for serum or plasma collection were spun in a centrifuge (Jouan CR3i) at 3000 rpm for 10 mins at 4°C. Plasma from lithium heparin tube and EDTA tube and serum from plain tube were transferred into the aliquot tubes and stored in the -80° C freezer at Human Nutrition Research Centre (HNRC) as quickly as possible for further analysis. Plasma from lithium heparin vacutainer tube was used for the measurement of NO_3^- (deproteinised prior to the chemiluminescence analysis using ethanol- details on the procedure are described in Chapter 3). Serum from plain vacutainer tube was used for the measurement of serum 25(OH)D concentration, and plasma from EDTA vacutainer tube was used for the measurement of ADMA.

5.3.9 Statistical analysis

Baseline characteristics of the participants are presented as arithmetic means ± SDs for numerical variables and counts and percentages for categorical variables. The assumption of normality for all variables were tested by visual inspection of the histograms and evaluating the p-value for the Shapiro-Wilks test (if the p-value was < 0.05, the data were considered as not normally distributed). For non-normally distributed variables, logarithmic transformations were applied and data are presented as median (with 25^{th} and 75^{th} percentiles). Two sided $p<0.05$ was considered statistically significant in all cases.

Mean concentration of serum 25(OH)D concentration were categorised into two groups i.e. below and above the cut-off for adequacy (<30 nmol/L and ≥30 nmol/L). Independent sample *t*-test was used to compare differences between two groups. Univariate General Linear Model was used to compare differences for the main dependent variables between age (A) and gender (G) groups (Fixed factor) and also explore their interaction (A*G). Bivariate correlations were used to assess the association between serum 25(OH)D concentrations with age, anthropometric measurements, blood pressure, physical activity level, PWV, whole-body NO production and biomarkers of vascular health. Correlation analyses were also adjusted for physical activity level and vitamin D supplementation. The association between whole-body

NO production with age, anthropometric measurements, blood pressure, physical activity level, pulse wave velocity and biomarkers of vascular health were explored. The Bland-Altman method (see Chapter 3 for detailed description of this approach) was used to assess the agreement between home and clinic resting BP measurements. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 22.0 program for Windows (SPSS Inc., Chicago, IL).

5.4 Results

5.4.1 Recruitment and baseline characteristics of the participants

Two hundred flyers containing the information of the study were sent out at Newcastle University targeting potential young male and female to participate in this study. The participants for the older group were sought from the volunteer database from previous studies conducted at the university. A total of 48 participants were contacted for the telephone screening interview. After screening, eight subjects did not meet the criteria (BMI >30 kg/m^2: three subjects; reluctant to have their blood taken: two subjects; the date and time not suitable: three subjects). Hence, 40 participants in total were enrolled in the study **(Figure 5.2)**. Visits were conducted between 8.45 am to 12.30 pm and the average visit duration was approximately 40 minutes per participant.

A total of 40 participants were recruited and completed the study {ten older male [68.5 years (60.7,70.0)], ten older female [62.0 years (55.7,68.2)], ten younger male [32.0 years (29.0,42.0)] and ten younger female [32.0 years (25.7,38.2)]. The study was well tolerated and 100% compliance was received for the saliva collection and blood pressure measurement at home. A summary of the participants' opinion and evaluation of the study are attached in **Appendix 5.7**.

Baseline characteristics of the participants demonstrate that, except for height and DBP, the two age groups were significantly different for age, anthropometric variables, SBP and heart rate ($P<0.05$). On average, the older group had higher BMI, FM, SBP and lower heart rate compared with the younger group of participants. The results also revealed a significant difference between male and female participants for height, weight, FM, SBP and heart rate **(Table 5.1)**.

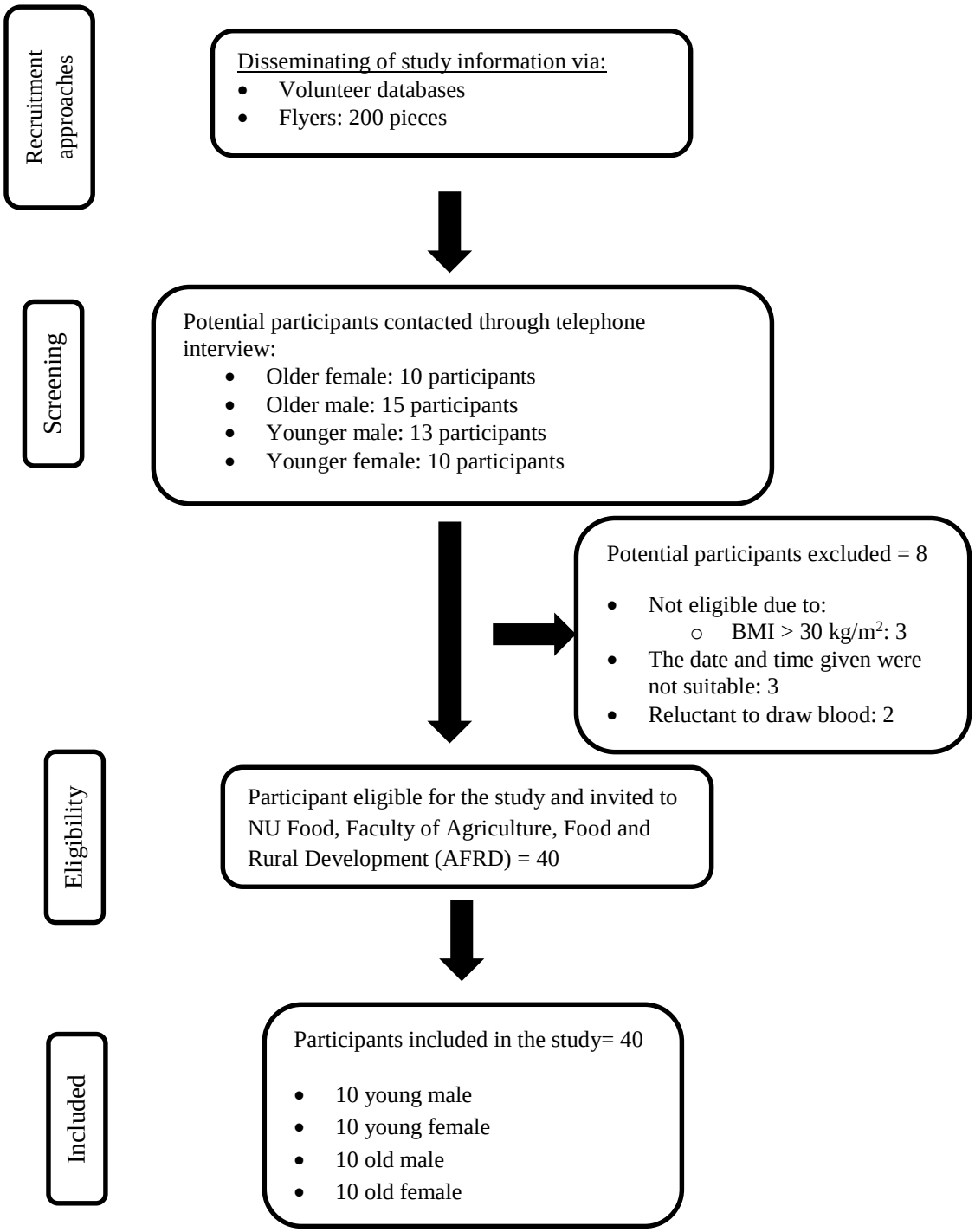


Figure 5. 2: Recruitment flow chart

Table 5. 1: Descriptive characteristics of the participants stratified by age and gender

	All	Younger (20 to 49 years old)		Older (50 to 70 years old)		P value
	N = 40	Male (n=10)	Female (n=10)	Male (n=10)	Female (n=10)	
			Mean (SD)			
Age (years) ¶	49.5 (31.0,66.5)	32.0 (29.0,42.0)	32.0 (25.7,38.2)	68.5 (60.7,70.0)	62.0 (55.7,68.2)	**A:< 0.001** G: 0.21 A*G: 0.94
Height (cm)	165.8 (9.1)	171.1 (8.87)	162.4 (4.2)	173.0 (6.3)	158.3 (7.6)	A: 0.39 **G: <0.001** A*G: 0.08
Body weight (kg)	69.8 (11.8)	69.5 (9.6)	61.6 (10.0)	81.8 (11.6)	66.7 (5.4)	**A: 0.04** **G: <0.001** A*G: 0.36
BMI (kg/m^2)	25.0 (3.4)	23.7 (2.8)	23.4 (3.6)	25.9 (3.8)	26.7 (2.2)	**A: 0.04** G: 0.67 A*G: 0.08
FM (kg) ¶	19.9 (10.9,23.3)	9.6 (8.6,15.3)	18.5 (13.1,21.2)	20.7 (13.2,23.4)	25.9 (21.4,26.6)	**A:< 0.001** **G: <0.001** A*G: 0.94
Clinic SBP (mmHg)	127.4 (17.2)	123.1 (8.1)	110.2 (10.9)	141.7 (14.3)	133.6 (15.8)	**A: <0.001** **G: <0.001** A*G: 0.48
Clinic DBP (mmHg)	74.9 (8.5)	76.2 (10.6)	68.7 (8.4)	78.3 (5.1)	76.4 (6.1)	A: 0.05 G: 0.07 A*G: 0.25
HR (bpm)	69.9 (9.8)	72.4 (8.1)	78.2 (9.8)	60.3 (5.0)	68.3 (6.4)	**A:< 0.001** **G: <0.001** A*G: 0.56

BMI, Body Mass Index; FM, Fat Mass; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; HR, Heart Rate. ¶ Reported as median (25^{th}, 75^{th} percentiles). Significant p values are shown in bold. Univariate Analysis of Variance was used to test the effect of age and gender on selected outcomes. Age, Age; G, Gender; A*G, Age times Gender interaction.

5.4.2 Nutrient intake and physical activity of the participants

The MEDE food frequency questionnaire was used to estimate the nutrient intake of the participants. Overall, we found that there was no significant difference in nutrient intakes between age and gender groups **(Table 5.2)**. However, older female participants appeared to have a higher consumption of CHO, fat, calcium, iron, sodium, vitamin C and vitamin D compared with the other groups but none of the Age*Gender interactions was significant. The intake of CHO and proteins was found to be within acceptable macronutrient distribution ranges for adults (CHO: 45-65%, protein: 10-35%) (Bates *et al.*, 2016) except for a slightly higher intake of energy from fat (36%).

The comparison of mean nutrient intakes by participants in our study with results from the National Diet and Nutrition Survey (2016) (NDNS), showed that participants in this study had higher consumption of CHO (342 g/day), protein (111 g/day), fat (113 g/day) and energy (2714 kcal/day) (total intake of these nutrients in NDNS for CHO, protein, fat and energy are 195 g/day, 67.7 g/day 60.9 g/day and 1608 kcal/day, respectively.) The intakes of calcium (1019 mg/day) and iron (18.5 mg/day) were also higher than the RNI (RNI for calcium and iron are 700 mg and 8.7 mg respectively). The vitamin D intake (3.39 µg/day) in this study was found to be similar to the result reported in Chapter 4 (3.82 µg/day), which is considerably less than the recommended RNI (10 µg/day) (SACN, 2016). Participants in this study had a high consumption of vitamin C (136 mg/day), which is almost four fold higher than the RNI for vitamin C (40 mg/day) (British Nutrition Foundation, 2016), and older females had the highest consumption (207 mg/day).

Total physical activity level (PAL) and walking PAL were highest in younger males **(Table 5.3)** but there were no significant Age or Gender effects or Age*Gender interactions for any measured component of PAL due to the large variability of the results.

Table 5. 2: Dietary intakes (amounts /d) by participants stratified by age and gender

	All	Younger (20 to 49 years old)		Older (50 to 70 years old)		P value
		Male	Female	Male	Female	
N	40	10	10	10	10	
			Mean (SD)			
Carbohydrate (g) ¶ (% in kcal)	342 (225,559) 48	261 (188,638) 45	432 (188,553) 53	277 (218,422) 44	346 (334,40) 45	A: 0.75 G: 0.34 A*G: 0.59
Fat (g) (% in kcal)	113 (59) 36	97 (68) 39	120 (72) 33	108 (51) 39	135 (51) 40	A: 0.65 G: 0.28 A*G: 0.88
Protein (g)¶ (% in kcal)	111 (79,153) 16	87.0 (52.4,162.3) 16	121.6 (75.6,175.8) 15	107.6 (72.2,135.5) 17	114.2 (98.4,150.1) 15	A: 0.83 G: 0.19 A*G: 0.95
Energy (kJ/day) ¶	2714 (1910,4071)	2774 (1681,4209)	2774 (1681,2774)	2624 (1793,3490)	2971 (2655,3665)	A: 0.65 G: 0.25 A*G: 0.84
Calcium (mg)	1019 (474)	822 (592)	1123 (443)	994 (473)	1215 (427)	A: 0.53 G: 0.14 A*G: 0.60
Iron (mg)	18.5 (9.1)	17.0 (14.3)	19.9 (8.5)	16.5 (6.8)	22.3 (8.1)	A: 0.99 G: 0.26 A*G: 0.86
Sodium (mg)	4250 (2201)	3172 (2390)	4616 (2501)	4695 (2285)	4869 (1794)	A: 0.31 G: 0.36 A*G: 0.25
Vitamin C (mg)¶	136 (84, 222)	89 (59,292)	151.1 (80.1,228.6)	116.9 (82.1,154.4)	207.0(163.7,252.9)	A: 0.31 G: 0.09 A*G: 0.39
Vitamin D (µg)¶	3.39 (2.13,4.95)	2.7 (1.83,5.59)	3.31 (2.46,5.57)	3.16 (2.1,4.9)	3.98 (1.68,4.39)	A: 0.85 G: 0.65 A*G: 0.66
Nitrate intake (mg/day) ¶	140 (107, 254)	124 (110,188)	107.0 (50.6,272.7)	150 (93,268)	214 (141,344)	A: 0.65 G: 0.94 A*G: 0.24

Univariate Analysis of Variance was used to test the effect of age and gender on selected outcomes. A, Age; G, Gender. A*G, Age times Gender interaction; ¶ Reported as median (25^{th}, 75^{th} percentiles).

Table 5. 3: Physical Activity Level (PAL) of participants stratified by age and gender

	All	Younger (20 to 49 years old)		Older (50 to 70 years old)		P value
		Male	Female	Male	Female	
N	40	10	10	10	10	
			Mean (SD)			
PAL walking (METs/wk)¶	1097 (693,2079)	1386 (594,3118)	1039 (693,1493)	1017 (470,2598)	1155 (668,2829)	A: 0.35 G: 0.98 A*G: 0.40
PAL moderate METs/wk)¶	60 (0.0,570)	140 (0.0,1260)	0.00 (0.0,90)	480 (180,720)	0.00 (0.0,3570)	A: 0.16 G: 0.39 A*G: 0.15
PAL vigorous (METs/wk)¶	0.00 (0.0,720)	420.0 (0.0,1440)	0.00 (0.0,540)	600 (0.0,1080)	0.00 (0.0,120.0)	A: 0.59 G: 0.46 A*G: 0.29
PAL Total (METs/wk)¶	1872 (1025,4200)	3348 (1683,4776)	1249 (866,2145)	1854 (1520,4331)	2142 (668,7628)	A: 0.33 G: 0.32 A*G:0.14

PAL, Physical Activity Level; MET, Metabolic Equivalent of Task; wk, week; Univariate Analysis of Variance was used to test the effect of age and gender on selected outcomes. A, Age; G, Gender; A*G, Age times Gender interaction.

¶ Reported as median (25^{th}, 75^{th} percentiles).

5.4.3 Sunshine exposure and vitamin D status

The relationships between habitual sunshine exposure and mean serum 25(OH)D concentration are summarised in **Table 5.4**. There was no relationship between time spent outdoors or time spent outdoors on weekdays and vitamin D status but serum 25(OH)D concentration were greater for those who spent more time outdoors (more than two hours) at the weekend.

Participant's ethnicity may have played a role in this study. The majority of the older participants were English (80 %; seven old female and nine old male), which have a fair skin type. The majority of younger participants were instead from Asia [15 participants (75%)], which were considered as having brown colour skin type. There was a different pattern of sunshine exposure between the older and younger groups. The younger group for both genders reported that they spend less time outdoors (between 15 to 30 minutes) especially during the weekdays. Older groups reported that they usually spend a longer time in outdoor activities (more than two hours). However, there were no significant associations between sunlight exposures, clothing, sunblock usage, skin type and serum 25(OH)D concentration.

Table 5. 4: Serum 25(OH)D concentrations by sunshine exposure variables as assessed by sunshine exposure questionnaire

Characteristics	Participants N (%)	25(OH)D concentration (nmol/L) (mean ±SD)	P value
1. How often are you outdoors for at least half an hour between 10 am to 3 pm during the sunny months (April – September)?			
• Less than once a week	5 (12.5)	46.1 **(**19.1)	
• 1 – 2 times a week	10 (25.0)	61.8 (20.2)	
• More than 2 times a week	9 (22.5)	58.5 (18.4)	0.11
• Everyday	16 (40.0)	42.5 (16.7)	
2. How much time would you spend outdoors on weekdays (Monday to Friday) during the sunny months (April – September)?			
• Less than 15 minutes	5 (12.50)	40.9 (18.8)	
• Between 15 and 30 minutes	10 (25.0)	47.4 (20.8)	0.33
• Between 30 minutes and 2 hours	9 (22.5)	48.0 (19.3)	
• More than 2 hours	16 (40.0)	56.9 (17.6)	
3. How much time would you spend outdoors on a weekend (Saturday - Sunday) during the sunny months (April-September)?			
• Less than 15 minutes	2 (5.0)	54.7 (19.0)	
• Between 15 and 30 minutes	12 (30.0)	41.6 (19.1)	**0.03**
• Between 30 minutes and 2 hours	20 (50.0)	49.9 (17.6)	
• More than 2 hours	6 (15.00)	69.0 (13.5)	

Characteristics	Participants N (%)	25(OH)D concentration (nmol/L) (mean ±SD)	P value
5. How would you deliberately wear less clothing so as to get direct sunlight on your skin (e.g. sleeveless tops shorts)?			
• Never	13 (32.5)	45.73 (21.0)	
• Rarely	20 (50.0)	49.58 (17.4)	
• Sometimes	6 (15.0)	60.29 (18.4)	0.27
• Usually	1(2.5)	73.9	
6. Sunblock usage			
• Yes	18 (45)	48.7	0.69
• No	22 (55)	52.2	
7. Skin type			
• Burns easily, never tans (white, very fair)	1 (2.5)	38.1	
• Burns easily, tans minimally (white, fair)	5 (12.5)	43.4 (21.3)	
• Burns moderately, tans gradually (cream white, fair)	13 (32.5)	51.6 (21.0)	0.78
• Burns minimally, tans well (brown, typical Mediterranean Caucasian skin)	13 (32.5)	54.5 (18.0)	
• Rarely burns, tans profusely (dark brown, mid-eastern skin types)	8 (20.0)	48.3 (19.1)	
8. When you are outdoors during the sunny months (April – September), do you stay in the sun or do you seek the shade?			
• I try to avoid staying in direct sunshine	13 (32.5)	52.1 (23.2)	
• I sometimes stay in the sunshine	20 (50)	49.3 (18.9)	0.91
• I enjoy staying in the sunshine	7 (17.5)	51.0 (12.4)	

Significant p values are shown in bold. One-way ANOVA was used to evaluate differences in 25(OH)D concentrations between groups.

5.4.4 Measurement of blood pressure (at research facility and at home)

Resting BP measurements were performed in two different locations; three measurements were recorded at the research facility and eight measurements were self-recorded at home by the participants (measured after each saliva collection). The average of the BP measurements was calculated and compared to evaluate the agreement between these two measurements protocols **(Table 5.6)**.

In general, the average SBP and DBP was within a normal range. A significant difference between age groups was found in SBP for the measurement recorded at the research facility ($p<0.001$). However, older participants had a higher SBP and DBP measured at the clinic and home compared to the younger group (seven older participants had a SBP greater than 140 mmHg). BP measurements at the research facility were found to be higher compared that the measurements performed at home.

These results are also illustrated in the scatterplots and B & A analysis **(Figure 5.3).** The regression analysis showed a good agreement between the two methods for SBP ($n = 40$, $r = 0.78$, $p<0.001$, **Figure 5.3 A**) but slightly weaker association for DBP ($n = 40$, $r = 0.64$, $p<0.001$, **Figure 5.3 C**). The B & A analysis provided a visual representation of the agreement between the two BP methods. The mean differences for SBP and DBP between the two methods were small [mean bias = 3.08 mmHg ($p=0.08$) and 2.90 mmHg ($p=0.01$)], respectively **(Table 5.5)**. The differences between the measurements were clustered within the 95% limits of agreement, with the exception for one point for SBP **(Figure 5.3 B)** and three points for DBP **(Figure 5.3 D)**. The regression lines fitted to the B&A analysis were not significant and therefore indicated a lack of differential bias with increasing SBP and DBP readings.

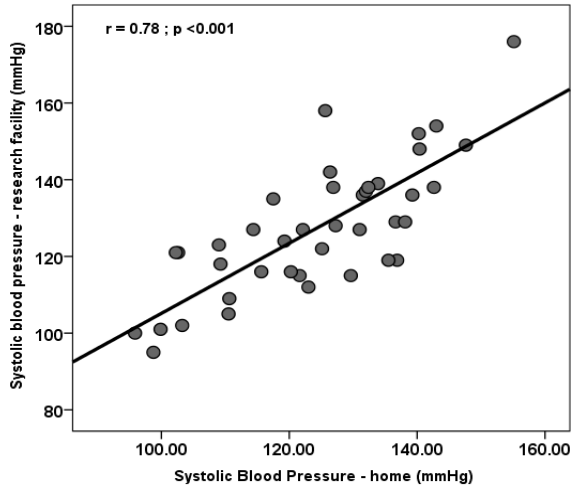
Table 5. 5: Correlation and agreement parameters of systolic (SBP, mmHg) and diastolic (DBP, mmHg) blood pressure measurements measured at the research facility and at home

	N	Mean Bias	Regression equation	r^2	t-test (p)	+2SD	Min	Max
SBP	40	3.08	y=-19.34+ 0.18x	0.06	0.08	10.88	-17.88	32.38
DBP	40	2.90	y=7.91-0.07x	0.005	0.01	7.35	-14.00	20.13

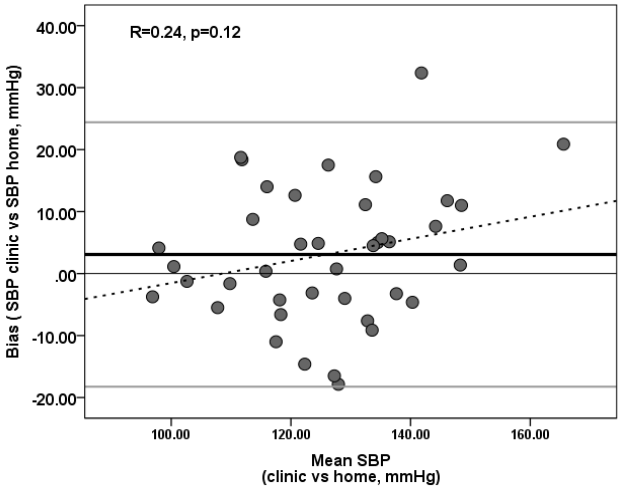
Table 5. 6: Measurement of blood pressure (at research facility and at home) stratified by age and gender

	All	Younger (20 to 49 years old)		Older (50 to 70 years old)		P value
		Male	Female	Male	Female	
N	**40**	**10**	**10**	**10**	**10**	
mmHg			**Mean (SD)**			
Mean SBP measured at research facility (mmHg)	127.4 (17.2)	123.1 (8.1)	110.2 (10.9)	141.70 (14.3)	134.60 (16.3)	**A: <0.001** **G: <0.001** A*G:0.48
Mean DBP measured at research facility (mmHg)	74.9 (8.5)	76.2 (10.6)	68.70 (8.4)	78.30 (5.1)	76.60 (6.4)	A: 0.05 G: 0.07 A*G: 0.25
Mean SBP measured at home (mmHg)	124.3 (14.7)	121.3 (12.9)	109.38 (10.7)	133.95 (11.8)	132.30 (9.4)	**A: <0.001** G: 0.60 A*G: 0.14
Mean DBP measured at home (mmHg)	72.0 (9.0)	74.5 (11.9)	67.23 (5.8)	73.62 (9.5)	72.72 (6.8)	A: 0.42 G: 0.15 A*G: 0.25

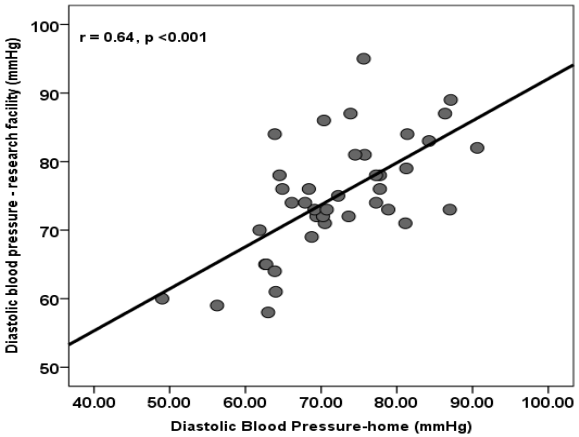
SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; Univariate Analysis of Variance was used to test the effect of age and gender on selected outcomes. A, Age; G, Gender; A*G, Age times Gender interaction. Significant p values ($p<0.05$) are shown in bold.



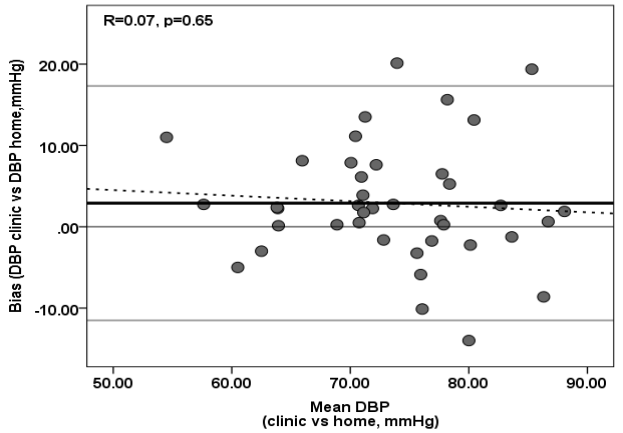
(A) Regression analysis of average systolic blood pressure (SBP, mmHg) measured at home and at research facility



(B) Bland-Altman analysis of mean systolic blood pressure (SBP, mmHg) measured at the clinic and at home



(C) Regression analysis of average diastolic blood pressure (mmHg) measured at home and at research facility



(D) Bland-Altman analysis of mean diastolic blood pressure (DBP, mmHg) measured at the clinic and at home

Figure 5. 3: Comparison of average systolic (SBP) and diastolic (DBP) blood pressure measured at the research facility and at home. (A) & (C): Linear regression analysis to evaluate the association between SBP and DBP measured at clinic and at home. (B) & (D): B&A plot to assess the agreement of BP measurements at the clinic and at home. Black horizontal line shows the mean difference (bold) and the ± 2S.D. range (fine, grey line). A regression line was fitted to the points (dashed black line) to evaluate differential bias.

5.4.5 Mean 25(OH)D concentrations

Serum 25(OH)D concentrations were significantly higher in older participants (Figure 5.4). Mean serum 25(OH)D concentrations for the older group were 66.4 nmol/L in males and 55.3 nmol/L in females, whilst in the younger group, the mean serum 25(OH)D concentrations were 39.6 nmol/L for male and 40.7 nmol/L for female participants **(Figure 5.4)**. In this study, we found that all older male participants had serum 25(OH)D concentrations of ≥ 30 nmol/L, and only one older female with less than 30 nmol/L. However, in the younger group, four females and one male participants had 25(OH)D concentrations <30 nmol/L **(Table 5.7)**. The maximum serum 25(OH)D concentrations in the old group (male and female) were 89.5 nmol/L and 86.4 nmol/L respectively, and the lowest for this group were 49.2 nmol/L and 29.8 nmol/L, respectively. For the young group, the maximum and minimum for young male were 65.8 nmol/L and 27.3 nmol/L, and for young female were 68.2 nmol/L and 17.3 nmol/L, respectively.

Eighteen (45%) participants in this study consumed vitamin D supplementation **(Table 5.7** and **Figure 5.5)**. The correlation between the vitamin D intake (from FFQ) with serum 25(OH)D concentrations was not significant in the total population (n= 40, r=-0.01, p=0.91) and after exclusion of participants taking vitamin D supplementation (n=22, r=-0.03, p=0.83).

Table 5. 7: Consumption of vitamin D supplementation stratified by age and gender

	All	Younger (20 to 49 years old)		Older (50 to 70 years old)	
		Male	Female	Male	Female
N	**40 (%)**	**10 (%)**	**10 (%)**	**10 (%)**	**10 (%)**
25(OH)D concentration (< 30 nmol/L)	6 (15)	1 (1)	4 (40)	0	1
• **Mean (SD) nmol/L**	24.83 (5.8)	27.34	22.96 (6.42)	-	29.80
25(OH)D concentration (≥ 30 nmol/L)	34 (85)	9 (90)	6 (60)	10(100)	9 (90)
• **Mean (SD) nmol/L**	55.08 (16.90)	41.06 (10.85)	52.53 (13.95)	66.47 (13.62)	58.16 (6.07)
Vitamin D Supplementation Usage					
Yes	18 (45)	2(20)	4(40)	7(70)	5 (50)
• **Mean 25(OH)D (SD) nmol/L**	67.8 (11.5)	57.6 (10.8)	60.1 (9.6)	73.4 (9.4)	70.3 (12.7)
No	22 (55)	8(20)	6(60)	3 (30)	5 (50)
• **Mean 25(OH)D (SD) nmol/L**	36.3 (10.1)	35.2 (5.2)	27.7 (8.9)	50.2 (0.9)	40.0 (10.9)

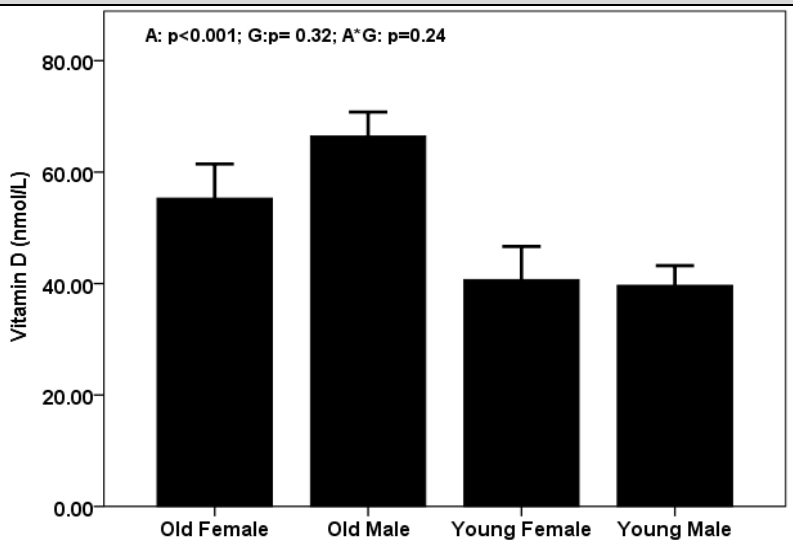


Figure 5. 4: Mean serum 25(OH)D concentrations stratified by gender and age group.
Univariate Analysis of Variance was used to test the effect of age and gender on selected outcomes. (A, Age; G, Gender; A*G, Age times Gender interaction)

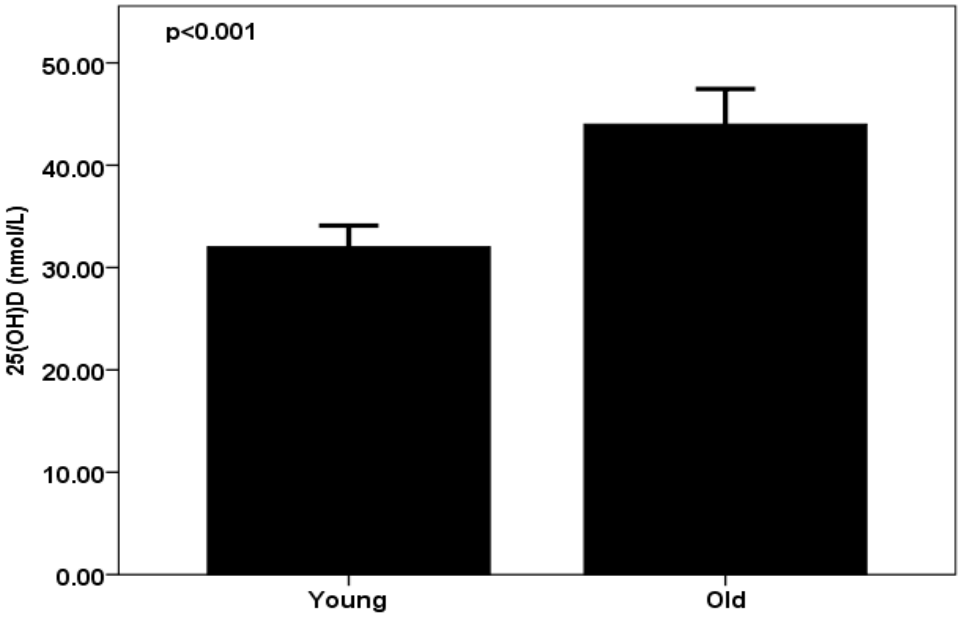
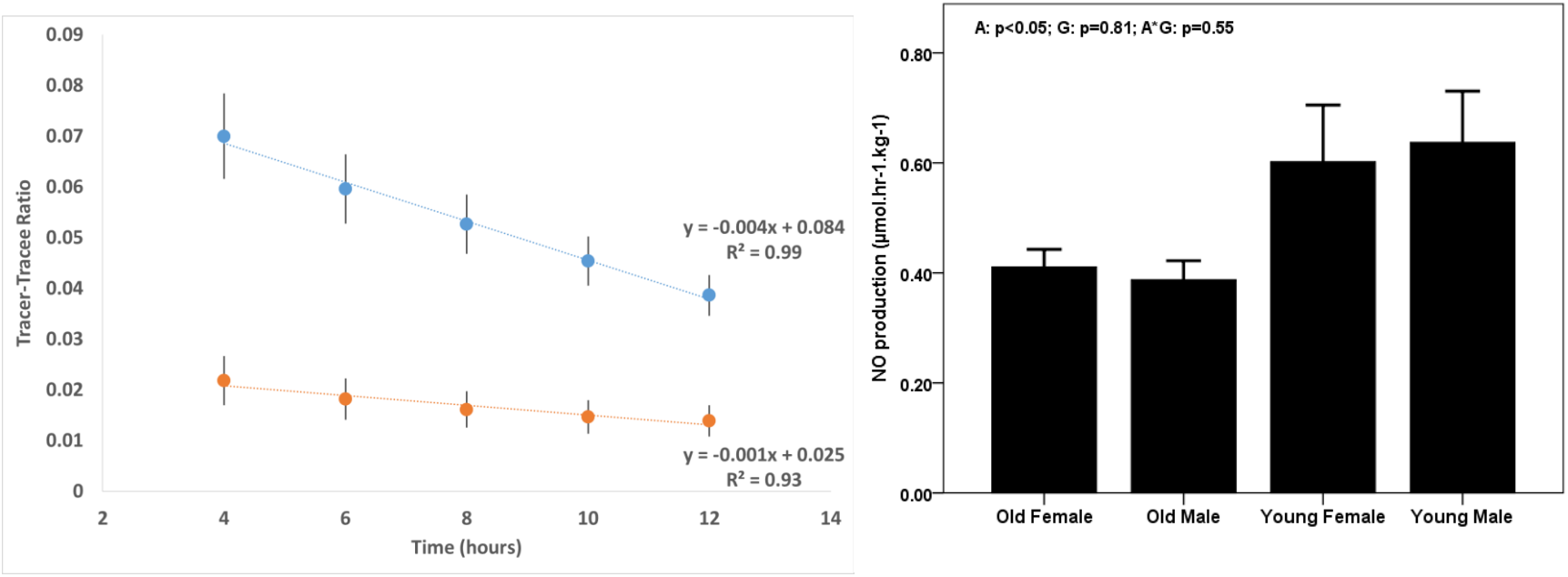


Figure 5. 5: Mean serum 25(OH)D concentrations in participants not taking vitamin D supplementation (n=22) stratified by age group. T-test for independent samples was used to compare the two groups.

5.4.6 Mean NO production stratified by gender and age group

Semi-logarithmic plots of saliva tracer/tracee ratio (TTR) were highly linear in both younger and older subjects ($r > 0.96$ in the period of 2 to 12 hours post dose). The younger group was characterised by a steeper slope of the regression line indicating a greater dilution of the tracer and hence a greater appearance of unlabelled nitrate compared with to the older group **(Figure 5.6 A)**. Male and female participants did not have a significantly different NO production rate ($p=0.82$). The values of NO synthesis ranged from 0.14 $\mu mol.hr^{-1}.kg^{-1}$ to 0.54 $\mu mol.hr^{-1}.kg^{-1}$ in the older group and 0.18 $\mu mol.hr^{-1}.kg^{-1}$ to 1.29 $\mu mol.hr^{-1}.kg^{-1}$ in the younger group. Mean NO production in younger people (0.61 $\mu mol.hr^{-1}.kg^{-1}$) was significantly higher than the older group (0.39 $\mu mol.hr^{-1}.kg^{-1}$) ($p=0.03$) **(Figure 5.6 B)**.



(A) **(B)**

Figure 5. 6: Tracer-tracee ratio of labelled/unlabelled salivary nitrate measured during the ONT test and mean NO production stratified by gender and age group

(A)Tracer-tracee ratio of labelled/unlabelled salivary nitrate measured during the ONT test and showed for younger (n=20, represented by blue line) and older (n=20, represented by orange line) participants. (B) Mean NO production stratified by gender and age group.
Univariate Analysis of Variance was used to test the effect of age and gender. (A, Age; G, Gender; A*G, Age times Gender interaction)

5.4.7 Pulse Wave Velocity

Mean PWV was higher in older (12.37 m/s) compared with younger (8.66 m/s) participants ($p<0.001$). Older men showed a mean PWV of 13.2 m/s, which is higher than the reference value for PWV (12 m/s), (Mancia *et al.*, 2007). The mean of PWV for old female subjects was 11.47 m/s. Seventeen (85%) old participants and two (10%) young participants had a value of 12 m/s or more for PWV measurements.

Likewise, the younger group demonstrated a lower mean PWV (9.33 m/s for male and 8.0 m/s for female), which indicates a better vascular health **(Figure 5.7)**. Overall, in this study, females had a lower value of PWV than males ($p<0.05$).

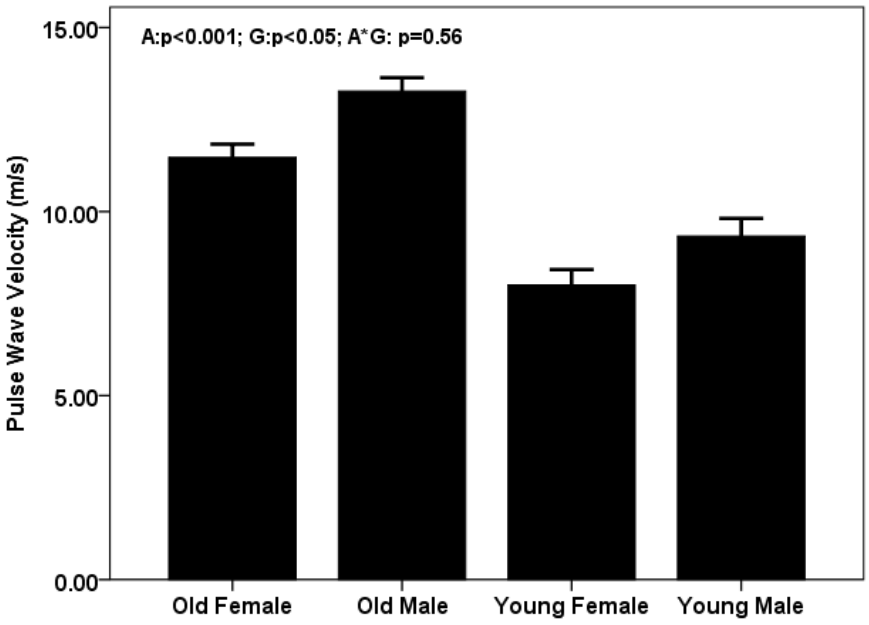


Figure 5. 7: Mean Pulse Wave Velocity stratified by gender and age group
Univariate Analysis of Variance was used to test the effect of age and gender.
(A, Age; G, Gender; A*G, Age times Gender interaction)

5.4.8 Measurements of ADMA, nitrate (plasma and urine) and plasma nitrite

There were no significant between-groups differences in plasma ADMA (p=0.50), plasma nitrate (p=0.42), urinary nitrate (p=0.86) and plasma nitrite (p=0.41) concentrations **(Table 5.8)**. The mean concentration of ADMA in this study was lower (89.01 ng/ml), compared to the previous cross sectional study reported in Chapter 4 (102.72 ng/ml). The same pattern was observed for plasma NO_3^- and urinary NO_3^- as the mean concentrations in this study were 14.5 µmol/L and 0.64 mmol/L - respectively; mean concentrations for - plasma and urinary NO_3^- were 34.67 µmol/L and 0.72 mmol/L in the previous cross sectional study measured in postmenopausal women. A large range of plasma NO_2^- concentration was observed in this study (81.0 nmol/L to 768 nmol/L), and the mean concentration was 300 nmol/L.

5.4.9 Age, anthropometric measurements, blood pressure, physical activity level, pulse wave velocity and biomarkers of vascular health stratified by 25(OH)D concentration

The stratification of the sample by vitamin D status showed that six participants in this study had 25(OH)D concentration less than <30 nmol/L. The description of age, anthropometric measurements, BP, PAL, PWV and biomarkers of vascular health stratified according to vitamin D status (<30 nmo/L and ≥ 30 nmol/L) is presented in **Table 5.9**. No significant differences were found between the groups except for height, weight, 25(OH)D concentrations and PWV. PWV measurements were higher in ≥30 nmol/L group (p=0.03).

5.4.10 Correlation between 25(OH)D with age, BMI, blood pressure, PWV, whole-body NO production and biomarkers of vascular health

Table 5.10 shows the unadjusted and adjusted (vitamin D supplementation and PAL) correlation between 25(OH)D concentration with age, BMI, BP, PWV, NO production and biomarkers of vascular health. Overall, there were positive, significant correlations between 25(OH)D concentrations (adjusted and unadjusted) with age (r=0.48, p<0.001), PWV (r=0.50, p<0.001) and negative, significant correlation with NO production (r=-0.37, p<0.001).

Table 5. 8: Measurements of ADMA, nitrate (plasma and urine) and plasma nitrite stratified by age and gender

	All	Younger (20 to 49 years old)		Older (50 to 70 years old)		P value
		Male	Female	Male	Female	
N	40	10	10	10	10	
			Mean (SD)			
ADMA (ng/mL)	89.01 (45.0)	96.1 (47.2)	85.7 (49.4)	82.3 (39.9)	91.7 (48.7)	A: 0.79 G: 0.97 A*G: 0.50
NO_3^-						
- Plasma (µmol/L)¶	14.5 (11.6,22.7)	15.90 (13.3,25.7)	13.81 (8.1,14.4)	18.64 (13.9,24.0)	12.1 (10.2,32.0)	A: 0.56 G: 0.18 A*G:0.42
- Urine (mmol/L)¶	0.64 (0.43,0.85)	0.84 (0.39,1.38)	0.70 (0.45,0.85)	0.58 (0.43,0.81)	0.55 (0.26,0.67)	A:0.19 G: 0.60 A*G: 0.86
Plasma NO_2^- (nmol/L)¶	256.5 (204, 377)	219 (156,528)	226 (152,345)	271 (227,361)	291 (248,399)	A: 0.20 G:0.79 A*G:0.41

PWV, Pulse Wave Velocity; ADMA, Asymmetric Dimethylarginine; NO, Nitric Oxide; Univariate Analysis of Variance was used to test the effect of age and gender on selected outcomes. A, Age; G, Gender; A*G, Age times Gender interaction.¶Reported as median (25^{th}, 75^{th} percentiles).

Table 5. 9: Age, anthropometric measurements, blood pressure, physical activity level, pulse wave velocity and biomarkers of vascular health stratified by 25(OH)D concentration status

	25(OH)D concentration		
	<30nmol/L	**≥30nmol**	**P value**
N	6	34	
Age (years)¶	38.5 (35.5,47.0)	55.5 (30.0,68.0)	0.49
Height (cm)	158.5 (7.2)	167.1 (8.9)	**0.03**
Body weight (kg)¶	62.8 (55.2,67.8)	69.3 (62.6,80.2)	**0.04**
BMI (kg/m^2)	24.4 (3.3)	25.1 (3.3)	0.65
FM (kg)¶	19.7 (12.3,24.6)	19.9 (10.2,23.4)	0.77
SBP (mmHg)	122 (20.4)	128.3 (16.8)	0.41
DBP (mmHg)	73.8 (6.7)	75.1 (8.8)	0.73
Home SBP (mmHg)	119.10(13.3)	125.23(14.9)	0.35
Home DBP (mmHg)	72.77(8.37)	71.91 (9.21)	0.83
25(OH)D concentration (nmol/L)	24.83 (5.8)	55.0 (16.9)	**<0.001**
PWV (m/s)	8.6 (1.20)	10.8 (2.3)	**0.03**
ADMA (ng/ml)	73.8 (46.8)	91.6 (44.8)	0.37
NO_3^- ¶			
- Plasma (µmol/L)	14.5 (12.2,26.1)	14.6 (11.3,22.3)	0.94
- Urine (mmol/L)	0.76 (0.64,1.25)	0.59 (0.37,0.82)	0.14
Plasma NO_2^- (nmol/L)¶	226.5 (146.2,376.7)	262.5 (216.0,380.2)	0.34
NO production ($\mu mol.hr^{-1}.kg^{-1}$)¶	0.52 (0.39,0.72)	0.46 (0.35,0.54)	0.42
PAL Total (METs/wk)¶	1329 (1039,4326)	2012 (967,4238)	0.50

¶Reported as median (25^{th}, 75^{th} percentiles)

BMI, Body Mass Index; FM, Fat Mass; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; 25(OH)D, 25-hydroxyvitamin-D; PAL, Physical Activity Level; MET, Metabolic Equivalent of Task; wk,, week; PWV, Pulse Wave Velocity; ADMA, Asymmetric Dimethylarginine; NO, Nitric Oxide; NO_3^- , nitrate; NO_2^-, nitrite. Significant p values are shown in bold. Independent sample *t*-test was used to compare the two groups.

Table 5. 10: Correlation between 25(OH)D concentration with age, BMI, blood pressure, PWV, whole-body NO production and biomarkers of vascular health

Variables	25(OH)D concentration (unadjusted)	25(OH)D concentration* (adjusted)
Age (years)	**r = 0.48 p <0.001**	**r = 0.48 p <0.001**
BMI (kg/m^2)	r = 0.22 p = 0.17	r = 0.05 p = 0.73
Clinic SBP (mmHg)	**r = 0.35 p <0.05**	r = 0.31 p = 0.06
Clinic DBP (mmHg)	r = 0.20 p = 0.21	r = 0.03 p = 0.82
PWV (m/s)	**r = 0.47 p<0.001**	**r = 0.50 p <0.001**
NO production ($\mu mol.hr^{-1}.kg^{-1}$)	**r = -0.44 p <0.001**	**r = -0.37 p <0.001**
Plasma NO_3^- (µmol/L)	r =0.04 p =0.77	r = 0.02 p = 0.88
Urinary NO_3^- (mmol/L)	r = -0.04 p =0.77	r = -0.15 p = 0.35
Plasma NO_2^- (nmol/L)	r =-0.10 p =0.95	r =0.06 p =0.71
ADMA (ng/ml)	r =0.09 p =0.55	r =-0.02 p =0.86

PWV, Pulse Wave Velocity; ADMA, Asymmetric Dimethylarginine; NO, Nitric Oxide; BMI, Body Mass Index; FM, Fat Mass; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; NO_3^- , nitrate; NO_2^-, nitrite
*This correlation was adjusted for age, vitamin D supplementation and physical activity level. Significant p values are shown in bold. r, Pearson's coefficient of correlation.

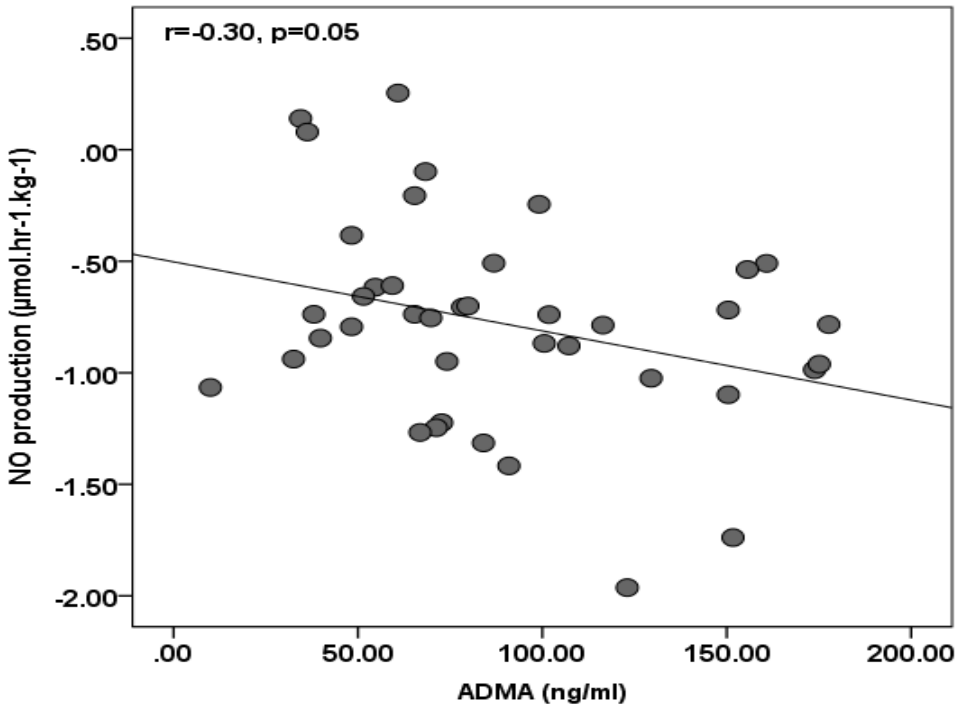
5.4.11 Correlation between NO production and PWV with anthropometric, blood pressure and biomarkers of vascular health.

There were positive, significant correlations between PWV with BMI, FM and home SBP (**Table 5.11)**. However, negative significant correlations were observed between NO production with home SBP and age. ADMA which showed an almost significant, inverse correlation with NO production (r=-0.30, p=0.05) **(Figure 5.8 A)**. There was also a significant, negative correlation between NO production and PWV (r=-0.37, p=0.01) **(Figure 5.8 B)**.

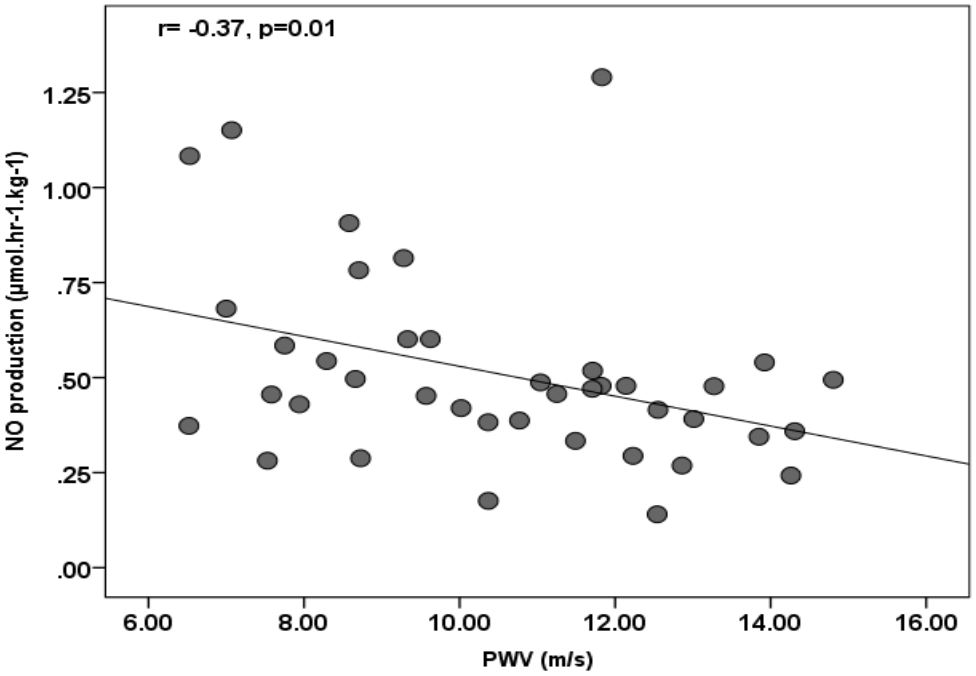
Table 5. 11: Correlation between NO production and PWV with anthropometric variables, blood pressure and biomarkers of vascular health

Variables	PWV	NO production
Age (years)	**r=0.80 p<0.01**	**r=-0.43 p<0.01**
BMI (kg/m^2)	**r= 0.54 p<0.01**	r=-0.23 p=0.14
FM (kg)	**r=0.47 p<0.01**	r=-0.22 p=0.15
Home SBP (mmHg)	**r=0.65 p<0.01**	**r=-0.41 p<0.01**
Home DBP (mmHg)	r=0.28 p=0.07	r=-0.12 p=0.45
Plasma NO_3^- (µmol/L)	r=0.14 p=0.36	r=0.15 p=0.35
Urinary NO_3^- (mmol/L)	r=-0.27 p=0.85	r=0.14 p=0.35
Plasma NO_2^- (nmol/L)	r=0.13 p=0.42	r=0.05 p=0.72
ADMA (ng/ml)	r=-0.03 p=0.84	r=-0.30 p=0.05

PWV, Pulse Wave Velocity; ADMA, Asymmetric Dimethylarginine; NO, Nitric Oxide; BMI, Body Mass Index; FM, Fat Mass; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; NO_3^-, nitrate; NO_2^-, nitrite. Significant p values are shown in bold. r, Pearson's coefficient of correlation.



(A) NO production vs ADMA



(B) NO production and PWV

Figure 5. 8: Regression analysis of (A) nitric oxide (NO) production with asymmetric dimethylarginine (ADMA) and (B) NO production with pulse wave velocity (PWV). r, Pearson's coefficient of correlation.

5.5 Discussion

5.5.1 Summary of findings

This study tested the hypothesis that ageing reduces whole-body NO production in normal and overweight, male and female participants. Additionally, we hypothesised that vitamin D may modify this association. We observed that NO production was significantly higher in the younger participants but no significant association was found with vitamin D concentrations. PWV was higher in the older group. There were no differences in whole-body NO production, nitrate, nitrite and ADMA for participants with or without adequate vitamin D status (25(OH)D concentration). However, there were positive, significant correlations between NO production and PWV with age, BMI, FM and SBP. These correlations were not explained by differences in 25(OH)D concentrations in either age groups.

5.5.2 Ageing, NO production and vascular health

NO is important to maintain vascular homeostasis, and lower production of NO has been associated with endothelial dysfunction. For example, in a study in a group of normal healthy volunteers aged between 20 and 79 years, PWV were significantly increased with age (Parikh et al., 2016). This was in line with a cross sectional study from a Swiss population of older adults that showed a significant association between age and arterial stiffness measured oscillometrically by pulse wave analysis (Endes et al., 2016). A study in Portugal observed a strong association between 'large artery damage' (defined as PWV >10 m/s) and older age in a cohort aged 60 to 70 years; 42% of these individuals had large artery damage (Cunha et al., 2015). Lower NO production may explain these age-related functional and structural vascular modifications (El Assar *et al.,* 2012). Several factors could contribute to the decrease in NO production and availability such as an increased oxidative stress, reduced availability of essential cofactors for eNOS, inhibition of eNOS by ADMA or accelerated NO degradation (Borsa *et al.,* 2012; Gradinaru *et al.,* 2015).

This is the first study in humans that quantified the impact of ageing on whole-body NO production measured using stable isotopic tracers. The rate of NO production in younger participants (0.61±0.3 $\mu mol.hr^{-1}.kg^{-1}$) was comparable with the aggregate rate of NO production in 18 healthy normal young people (mean age 34 ±13 years) subjects reported in previous studies using stable isotope tracers (0.63 ± 0.30 $\mu mol.hr^{-1}.kg^{-1}$) (Siervo *et al.*, 2011b). This provides further support for a reference normal range of NO production in healthy young individuals measured in independent studies and using different isotopic methods. Forte *et al*

(1997) reported significantly higher NO production measured by stable isotopes in hypertensive women compared with men (Forte *et al.*, 1997) but we found no evidence of a gender difference in whole-body NO production in this study.

Estimates of whole-body NO production in this study was similar to NO production rates for healthy middle-aged and older obese individuals measured using the same ONT method several years ago. The estimation of NO production from different studies was summarised in **Figure 5.9**.

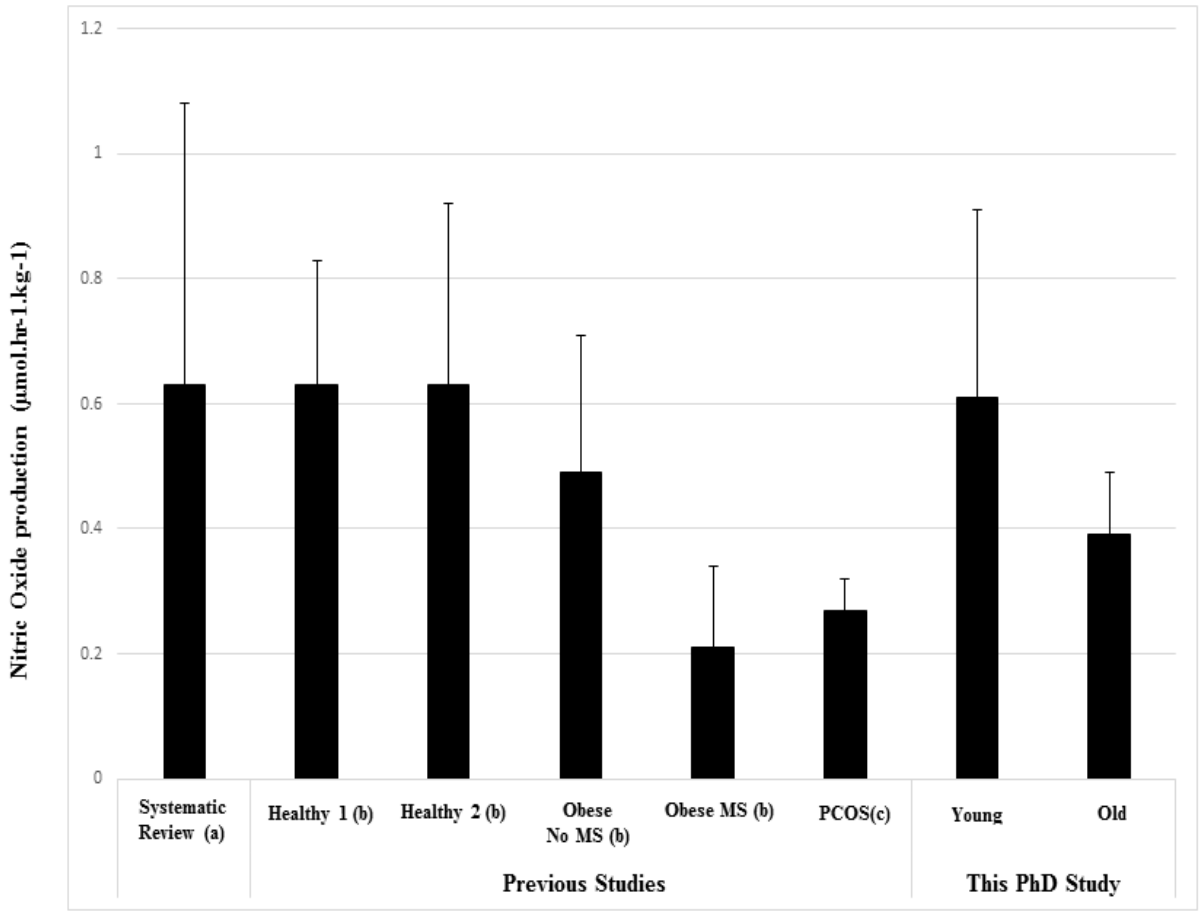


Figure 5. 9: Estimation of NO production rates from different studies using ONT method

Data obtained from :(a):(Siervo *et al.*, 2011c); (b)(Siervo *et al.*, 2011b), (c) PhD thesis, Virginia Tomatis, Cambridge University 2015 (Personal communication from Dr Siervo).
Mean age for study (a): Not reported, (b): Healthy 1 = 34 ±13 years; Healthy 2 = 27 ±11 years; Obese No MS = 48 ±10 years (c): PCOS = 28 ±10 years
MS, Metabolic syndrome; PCOS, Polycystic ovary syndrome

This is the first study to report an inverse association between whole-body NO production (measured using stable isotopes) and PWV, which may provide further support for the hypothesis that lower NO is associated with structural modifications of the arterial wall. Whole-body NO production was also inversely associated with SBP (measured in the clinic and at

home) which confirmed the results from two other studies using stable isotope tracers to measure NO production (Forte *et al.*, 1997; Siervo *et al.*, 2011b). Plasma ADMA concentrations showed a tendency to be associated with NO production in this study which confirmed the results of a previous study employing the same ONT method. These results may be explained by the characteristics of the population (overall healthy) and the associations with NO production should be tested in conditions with known increase in ADMA concentrations such as chronic kidney disease or heart failure. There was a non-significant trend for higher ADMA concentrations between the two age groups, which could be due to both groups being relatively healthy phenotype. However, a study by (Kielstein et al., 2003) revealed that plasma ADMA concentration (measured with high-performance liquid chromatography (HPLC)) was significantly higher ($p<0.05$) in older (2.77±0.20 μmol/L) than in young healthy subjects (1.30±0.11 μmol/L). The latter finding is in agreement with the significant positive correlation ($r=0.54$, $p<0.0001$) between age and ADMA observed in a random population sample in Tokyo (Miyazaki *et al.*, 1999).

5.5.3 Impact of vitamin D, NO production and vascular health

Higher serum 25(OH)D concentration were found to be associated with lower NO production and greater systolic BP and PWV. In particular, those with 25(OH)D concentration ≥30 nmol/L have higher PWV compared with participants with lower 25(OH)D concentration. These results are contrary to our hypotheses and also to what reported in the previous cross sectional study reported in Chapter 4, where PWV was significantly higher in those with 25(OH)D less than 30 nmol/L. Participants with an lower 25(OH)D concentrations were characterised by a slightly higher NO production (0.52 μmol.hr^{-1}.kg^{-1}) compared with participants with higher 25(OH)D concentrations (0.46 μmol.hr^{-1}·kg^{-1}). These differences and associations were maintained after the exclusion of participants taking vitamin D supplements or after adjustment of the analysis for vitamin D supplementation, age or physical activity levels. Similarly, ADMA concentrations were found to be lower in the insufficient compared to the higher 25(OH)D concentrations (73.8 vs 91.6 ng/ml). However, a recent cross sectional study conducted on 269 men and 382 women Korean elderly population demonstrated that the mean ADMA concentration was significantly higher in the insufficient 25(OH)D group (17.92 ng/ml, $p=0.001$) compared with the sufficient 25(OH)D group (15.87 ng/ml) (Choi *et al.*, 2017). In this study, they stratified the patients by 25(OH)D concentration into three groups according to serum 25(OH)D: sufficient (≥30 ng/mL, n=25), insufficient (10-<30 ng/mL, n=516), and deficient (<10 ng/mL, n=110). The measurement of ADMA concentration was assayed by HPLC. The mean ADMA concentration was significantly higher in the insufficient 25(OH)D

concentration (0.665 μmol/L, p=0.001) and the deficient 25(OH)D concentration (0.734 μmol/L, p<0.001) compared with the sufficient 25(OH)D concentration (0.589 μmol/L).

The hypotheses on a positive association between 25(OH)D concentration, NO production and measures of vascular health were mechanistically based on the results from animal or human studies linking multiple possible pathways through which 25(OH)D concentration potentially influence NO availability. For example, in diabetic mice lacking VDR study, vitamin D suppressed renin-angiotensin system (RAS) activity by suppression of renin gene transcription (Deb et al., 2009), which leads to reduced angiotensin II and also theoretically to greater NO production, less NO destruction, and increased NO availability. Similarly, a study done in mice with functionally inactive VDR reported that serum 25(OH)D concentration enhances the production of enzyme eNOS in the endothelium and enhanced NO production (Andrukhova et al., 2014). A study in 419 elderly in Ankara, Turkey by (Kutlay et al., 2014) showed an inverse association between PWV and 25(OH)D concentration. In their study, three cut of points for 25(OH)D concentration were used [1st (< 20 ng/ml), 2nd (20–30 ng/ml) and 3rd (> 30 ng/ml)], and there were significant differences of 25(OH)D concentration between all the groups. Surprisingly, this study seems to have an opposite pattern of association with both NO production, blood pressure and PWV, which may suggest the existence of possible compensatory mechanisms that may sense the decrease in 25(OH)D concentration and potentially trigger positive feedback signals to enhance NO production. However, this alternative hypothesis remains highly speculative and needs to be tested in cellular and animal studies.

In addition, no significant correlations were observed between serum 25(OH)D concentration and NO metabolites (plasma NO_3^- (r=0.04; p=0.77), urinary NO_3^- (r=-0.44; p=0.77), and plasma NO_2^- (r=-0.10; p=0.95), even after adjustment for vitamin D supplementation and physical activity level. Similarly, a previous study conducted in 66 obese Caucasian children aged 7 to 14 years demonstrated no significant association of 25(OH)D concentration with plasma nitrite + nitrate and urinary nitrate concentrations (Codoner-Franch *et al.*, 2012). In this study, serum 25(OH)D concentration was quantified by electrochemiluminescence immunoassays, and NO_3^- levels in plasma and urine were measured by spectrophotometrically using the Griess reaction. In contrast, a significant association between 25(OH)D concentration with NO metabolites (NO_3^- and NO_2^-) was found in a cross sectional study in obese African-Americans (Valiña-Tóth *et al.*, 2012). In the study, serum 25(OH)D concentration was determined using liquid chromatography-tandem spectrometry, and NO_3^- and NO_2^- was

determined using Cayman's Nitrate/Nitrite colorimetric assay kit. Therefore, these discrepancy results between studies may be related to the study participants characteristic, method of measuring serum 25(OH)D concentration and NO metabolites.

5.5.4 BP measurements and nitrite as a biomarker of NO production

This study demonstrated a significant difference between the measurements of BP recorded at the clinic and at home. The measurement of SBP was found to be higher when measured at the clinic, especially in the older group. The difference may be simply explained by the psychological and emotional responses triggered by the measurement of BP in a clinical environment which defines the characteristics of the white-coat syndrome. BP methods that can minimise these effects are important from a diagnostic perspective and home monitoring of BP has been currently suggested as a valid and sensitive method to diagnose and monitor changes in BP (Franklin *et al.*, 2016; Mancia *et al.*, 2017). The measurement of BP proposed as part of the ONT protocol was easy to conduct and the lower BP readings were probably explained by the more comfortable and relaxed conditions experienced by each participant. A similar drop in BP readings was reported in a study conducted in 45 elderly patients (≥70 years), which revealed a significant difference between the BP measurements during hospital admission and home follow up (systolic from 133.5±15.6 to 126.2±14.4mmHg, P=.008; diastolic from 71.0±9.0 to 68.3±8.6 mmHg, P=.046) (Cappelleri, 2017). For our perspective, the significant association of the home BP readings with NO production and other measurements of vascular health provide further support on the feasibility of this BP method in future studies using the ONT method for the assessment of NO production.

NO_2^- is widely accepted as a surrogate marker for NO, and has been shown to be a circulating storage pool for NO (Calvert and Lefer, 2010). In addition, NO_2^- can be rapidly converted to NO under ischemic conditions (Sindler *et al.*, 2014). However, it is not clear whether NO_2^- reflect regional endothelial function as assessed by brachial artery FMD. A study by Casey *et al*, 2007 demonstrates that systemic basal plasma levels of NO_2^- correlate with brachial FMD in young adults. This relationship suggests that brachial FMD and/or plasma NO_2^- may be used as markers of peripheral endothelial function in the young population (Casey *et al.*, 2007). In contrast, ADMA is an endogenous competitive inhibitor for the NO synthesis (Duygu *et al.*, 2006). Evidence indicates the inverse relationship between ADMA and NO_2^-/NO_3^- (Cevik *et al.*, 2006; Eleuterio *et al.*, 2013). This in in line with a study by (Haberka *et al.*, 2009) that revealed the inversed association between ADMA with FMD (0.387; p = 0.012).

However, in this study, no association was found between NO_2^- with biomarkers of vascular health, which may be due to several factors such as small sample size, general characteristics of the participants and methods of biomarkers measurement. The lack of associations in this study leaves room for further work and research regarding the relationship between NO metabolites and vascular health.

5.5.5 Strength and limitations

To my knowledge, this is the largest study using stable isotopes to investigate associations between vitamin D, ageing and whole body NO production. The compliance of participants with this study protocol was excellent and all participants completed the study. The saliva ONT method was well tolerated, feasible and, with further validation studies in clinically relevant populations, could potentially become a reference method for the quantitative assessment of NO production in humans.

This study has a few limitations. The cross-sectional design is unable to provide information on the causality and direction of the associations between ageing, vitamin D status and NO production. Despite being the biggest study of its kind, the study had also a limited sample size (40 participants) and recruited a selected group of 20 healthy younger and 20 older participants. Therefore, the representativeness of the findings might be limited which may also be influenced by heterogeneous ethnic background of the participants. The limited sample size prevented from the exploration of a full interaction analysis between age with either gender or vitamin D levels which needs to be explored in future studies. In addition, the small number of participants with lower vitamin D status did not allow for a more detailed analysis on the interaction between age and vitamin D status.

The number of participants with insufficient vitamin D status (assessed by 25(OH)D concentration) was small as the majority of the participants (85%) were in the higher 25(OH)D groups, which might potentially have contributed to the unexpected associations found in the study. Measurements of 25(OH)D concentrations in this study were performed using immunoassays. Unlike chromatographic methods, immunoassays do not measure vitamin D_3 and vitamin D_2 independently, and this is a well-recognised limitation of immunoassays. The importance of being able to quantify both metabolites of vitamin D independently is becoming increasingly important with the evidence that vitamin D_3 is more biologically active than vitamin D_2 (Tripkovic et al., 2012).

The usage of vitamin D supplementation might have contributed to the confounding factor in this study as most of the older people in this study consumed vitamin D supplementation (60%), compared to only 30% in the younger group. This explained the discrepancy of 25(OH)D status between the two age groups. However, the exclusion of subjects taking vitamin D supplements did not modify the results. The study was conducted during the summer season and we can therefore disregard the seasonal variability in vitamin D exposure as a confounding factor.

5.5.6 Conclusions

In conclusion, we found that whole-body NO production is significantly lower in older people. In addition, the associations between NO production and systolic BP and PWV provide further support for the feasibility and sensitivity of the ONT stable isotopic method for the assessment of whole-body NO production in humans. Unexpectedly, this study found significant lower NO production in participants with higher serum 25(OH)D concentrations. These results are contrary to the original hypotheses stipulated in this project and therefore further studies are needed to confirm these findings and to clarify the pathophysiological mechanisms underlying these associations.

Chapter 6: General Discussion and Conclusions

6.1 Overview of the PhD work

A low vitamin D status is a common public health problem worldwide and it is associated with both musculoskeletal and non-musculoskeletal effects (Palacios and Gonzalez, 2014). However, the association between vitamin D deficiency and non-musculoskeletal conditions is not adequately established and the effects of vitamin D supplementation remain debatable (Hossein-nezhad and Holick, 2013). Vitamin D influences vascular and metabolic processes including endothelial function (EF), a surrogate marker and predictor of cardiovascular disease risk. The evidence for a causal relationship between vitamin D and cardiovascular risk is inconsistent and inconclusive, and these discrepant results have raised further questions about the relationship between serum 25(OH)D concentration (the accepted marker of vitamin D status) and EF.

Vitamin D has been suggested to have potential role in preventing CVD and type 2 diabetes mellitus (Danik and Manson, 2012). The individual RCTs have been designed following the report of significant associations between low vitamin D status and cardiovascular risk in cohort studies (Martins *et al.*, 2007; Dobnig *et al.*, 2008; Hintzpeter *et al.*, 2008; Hypponen *et al.*, 2008) and mechanistic cellular and animal studies reporting a role of vitamin D in the regulation of NO production. However, the results of individual studies have been inconsistent and meta-analysis of RCTs did not support the beneficial effects of vitamin D supplementation on CVD risk (Wang *et al.*, 2010; Ford *et al.*, 2014). Similarly, systematic review and meta-analysis evaluating the effects of vitamin D supplementation and endothelial function in RCTs did not support a protective role (Alyami *et al.*, 2014; Hussin *et al.*, 2017).

The results from two independent observational studies carried out as part of this PhD project showed that serum 25(OH)D concentration had weak and inconsistent associations with physiological and biochemical markers of EF (i.e. PWV and ADMA). Perhaps, more importantly, the results presented in Chapter 5 show an inverse association between vitamin D status (serum 25(OH)D concentration) and whole-body NO production. While these cross-sectional studies have significant limitations (discussed below), they have used a comprehensive panel of methods, including stable isotopes, to provide reliable measurements of EF and NO availability.

This PhD project has investigated the association between vitamin D intake and status, EF and biomarkers of NO availability. First, a systematic review and meta-analysis was conducted to

evaluate the effects of vitamin D supplementation on markers of EF. A laboratory experiment also was conducted to improve the measurement of NO_2^- and NO_3^- using ozone-based chemiluminescence and to evaluate the agreement between circulating 25(OH)D concentrations measured in serum and using dry blood spots. Two independent cross-sectional studies were conducted to investigate whether serum 25(OH)D concentrations significantly associated with physiological and biochemical markers of EF in post-menopausal overweight and obese women (study 1) and to evaluate the association between age and whole body NO production measured using stable isotope tracers in healthy young and old individuals (study 2).

6.1.1 Effects of vitamin D supplementation on Endothelial Function

The first phase of the PhD included a systematic review and meta-analyses of RCT to assess the effects of vitamin D supplementation and serum 25(OH)D concentrations on EF (discussed in Chapter 2). The analyses also examined whether the effect size was modified by health status, study duration, dose, route of vitamin D administration, vitamin D status (baseline and post-intervention), BMI, age and type of vitamin D (vitamin D_2 or vitamin D_3). Overall, there was no evidence for an effect of vitamin D on EF. However, subgroup analysis showed a significant improvement of EF in diabetic subjects and in individuals with a higher BMI following vitamin D supplementation (daily doses varying from 1000 IU/day to 5000 IU/day). The significant benefit of vitamin D supplementation in diabetics and in those with higher BMI may indicate a role for the insulin pathway in modulating the effects of vitamin D on vascular function.

6.1.2 Methods for measuring biomarkers of endothelial function and serum 25(OH)D concentration

A summary of the methods and techniques for the measurement of NO production have been provided in Chapter 3. In this chapter, a detailed description of the different types of laboratory experiments has been provided for the measurement of NO_2^- and NO_3^- in plasma and urine samples. In particular, studies were conducted to investigate whether different deproteination procedures of plasma samples (using ethanol or methanol) influenced the measurement of plasma NO_2^- and NO_3^- concentration using ozone-based chemiluminescence. The finding showed that two deproteination methods had a negligible, non-significant influence on the measurement of NO_3^- concentration in plasma samples. However, the procedure using methanol resulted in significantly higher NO_2^- concentrations compared with use of ethanol. In Chapter 3, the agreement between two methods (conventional method using chemiluminescence immunoassay (CLIA) on liquid serum collected by venepuncture and dry blood spot (DBS) method) were investigated for the analysis of serum 25(OH)D concentrations.

The collection and analysis of DBS represent a simple and efficient method that may facilitate the measurement of serum 25(OH)D concentrations in large population based studies. Several studies have reported significant agreement between serum 25(OH)D concentrations measured by the conventional method and DBS (Heath et al., 2014; Sakhi et al., 2015; Hoeller et al., 2016). However, in this study, DBS has been found to be significantly over-estimate the serum 25(OH)D concentration compared with the conventional method. The 25(OH)D concentration measured by DBS was found to be more than two-folds, which is doubtful. As these unusually high 25(OH)D result is atypical for North-East women (SACN, 2016), this study did not recommend the usage of DBS and it seems that the results from CLIA estimations are more precise. However, further validation studies are needed to establish the agreement between these two methods.

The measurements of serum 25(OH)D concentration substantially varies depending on the methods of measurements and type of assay. There are two major types of assay, i.e. binding assay (CLIA, RIA and binding protein assay), which use a kit or an automated clinical chemistry platform, and chemical assay (HPLC and LC-MS/MS) and this method is able to separate the estimation of vitamin D_3 and D_2 concentrations (SACN, 2016). Significant differences in the results of 25(OH)D were observed among the various assays, and interpretation of the data from the study was also complicated by the fact that measurement of serum 25(OH)D concentration is affected by inter-assay differences. Standardization and harmonization of 25(OH)D measurements are therefore urgently needed.

6.1.3 Association of serum 25(OH)D with physiological and biochemical markers of endothelial function

This cross sectional study investigated the association of serum 25(OH)D concentration with physiological and biochemical markers of EF in overweight and obese post-menopausal women. Overall, the results showed no evidence for a significant association between serum 25(OH)D concentration with biomarkers of endothelial function, blood pressure, age and BMI (discussed in Chapter 4). However, PWV was found to be higher in individuals with lower serum 25(OH)D concentration. In addition, ADMA concentration was higher in the lower serum 25(OH)D concentration group, but this difference was not quite statistically significant ($p=0.07$). Overall, these results provided a weak support for the hypothesis that serum 25(OH)D concentration is associated with vascular EF among overweight and obese, post-menopausal women. The initial plans for this study also included a comparison between two different methods of estimating 25(OH)D concentration using 1) the conventional method (based on venepuncture) and 2) a more novel method based on analysis of dry blood spots (DBS).

However, because the analyses of DBS were doubtful (see Chapter 3) in Chapter 4, only results from the conventional method has been used.

This provided the basis for a more detailed study which was designed to explore the association between whole-body NO production measured by a non-invasive stable isotopic study and to evaluate the associations with PWV and ADMA concentrations in a population of younger and older healthy individuals (reported in Chapter 5 – see below).

6.1.4 Ageing, NO production and serum 25(OH)D

A second cross sectional study was performed on 40 healthy, normal weight and overweight males and females within two age brackets (20-50y, 50 - 65y) (discussed in Chapter 5). This final phase of the PhD project was done to investigate the associations between whole-body NO with PWV, ADMA, serum 25(OH)D and to investigate whether these associations are modified by age. The results revealed that whole-body NO production decreases with age and was also significantly associated with PWV and systolic blood pressure. Surprisingly, this study showed significant inverse associations between serum 25(OH)D concentrations and whole-body NO production, PWV and systolic blood pressure. The results from this study provide no support for our primary hypothesis of a direct association between serum 25(OH)D concentration and NO production and call for further investigations to confirm these results in populations with larger samples size and more detailed assessment of cardiovascular phenotypes. Additional studies are therefore needed to clarify the supposed causal relationship between vitamin D and NO.

6.2 Defining an appropriate 25(OH)D concentration

There are ongoing discussions and debates concerning the serum 25(OH)D concentration cut off levels for defining vitamin D sufficiency/insufficiency. IOM concluded that the RDA for those aged 1–70 years were 600 IU/d whilst for those aged 71 years and older, the RDA is 800IU/d. These were the intakes considered necessary to ensure that the corresponding serum 25(OH)D concentration was at least 20 ng/ml (50 nmol/liter) which is the concentration required for good bone health in 97.5% of the population (Ross *et al.*, 2011). On this basis, individuals with the serum 25(OH)D concentration <30 nmol/L are classified as deficient, and those with serum 25(OH)D concentration of 50 nmol/L and above are classified as sufficient. However, many researchers and expert groups including the Endocrine Society Task Force on Vitamin D and the International Osteoporosis Foundation do not accept this cut off point and higher intakes of vitamin D, between 800 to 2000 IU per day, achieved through supplementation or dietary intake have been recommended (Buck Institute for Research on Aging, 2016). The

Endocrine Society Task Force concluded that *those with a cut-off level of 50nmol/L are identified as vitamin D deficient, and to maximise the effect of vitamin D on calcium, bone and muscle metabolism, serum 25(OH)D concentration 'should exceed 75 nmol/L'* (Holick, 2011).

SACN (2016) has established a RNI for vitamin D of 10 µg/day (with assumption of minimal sunshine exposure) which is based on the conclusion that serum 25(OH)D concentration below 25 nmol/L lead to increased risk of poor musculoskeletal health (SACN, 2016). Most authorities base their cut off concentration for 25(OH)D on what is considered as the adequate concentration for protecting musculoskeletal health. SACN (2016) could not draw any inference about the relationship between serum 25(OH)D concentration and risk of developing CVD.

Given the uncertainties about the appropriate cut off points for 25(OH)D concentration, 30 nmol/L as the cut off point for vitamin D adequacy has been used in this cross sectional studies. The rationale behind this is that a serum concentration of 30 nmol/L was considered to be consistent with the lower end of requirements as reported by IOM (Ross *et al.*, 2011).

6.3 Strengths and limitations of the PhD work

This PhD study has investigated for the first time, the association between whole-body NO production measured using a non-invasive stable isotopic method with PWV and ADMA concentrations. The measurements of NO_2^- and NO_3^- in this study used chemiluminescence, which is considered as the reference method of measuring of NO_2^- and NO_3^- in plasma (Pelletier *et al.*, 2006). The measurement of whole body NO production was done using GCMS, which has been reported to be accurate, specific and highly sensitive for the determination of NO synthesis (Tsikas *et al.*, 2000).

However, there are some limitations associated with the study which need to be addressed in future studies. The two studies done in this PhD were designed as cross sectional studies based on recruitment of convenient samples, thereby making it difficult to determine the causal relationships. Furthermore, in the second cross sectional study, apart from the small sample size, the participants were recruited without the exclusion of vitamin D supplementation, which might have been a confounding of the associations between serum 25(OH)D concentrations and health outcomes. The participants in the studies were overall healthy and did not represent the total population in the UK. Lastly, the cross sectional studies in this PhD used single measurements of serum 25(OH)D concentrations as indicators of vitamin D status; thus this may not accurately reflect long term vitamin D status.

6.4 Conclusions and Future Work

The objectives of the PhD programme have been achieved successfully by conducting a series of reviews, laboratory analyses and human studies to investigate whether serum 25(OH)D concentration is associated with EF and *in vivo* whole-body NO production. The results generated in this thesis have highlighted several further questions about the relationship of serum 25(OH)D concentration with EF in humans. New hypotheses to be explored in future work are detailed below.

In the first cross sectional study, the target population was on overweight and obese postmenopausal women aged between 50 to 70 years old. The hypothesis that higher serum 25(OH)D concentration may improve PWV and ADMA measurement remains to be tested in prospective studies. Such studies would benefit from recruiting a similar population group (overweight and obese postmenopausal women aged between 50 to 70 years old) with a wide range of 25(OH)D concentration in which those with low vitamin D status are well-matched in all other respects with those with higher vitamin D status. In the second cross sectional study, for the first time, the effects of ageing on whole body NO production using stable isotopes was investigated. This study revealed a surprising inverse association between serum 25(OH)D concentration and NO production.

The results generated in this thesis have highlighted several potential of synergistic interaction of serum 25(OH)D concentration and EF that may have implications for future research. For example, there was evidence of positive effects of vitamin D supplementation in EF for obese individuals and in diabetic patients. Moreover, the first cross sectional study in this PhD revealed increased PWV and higher concentrations of ADMA in those with vitamin D concentrations < 30 nmol/L. Even though the second study of this PhD did not find a significant association of vitamin D with NO production, NO production was found to be significantly lower in older individuals and was significantly associated with systolic blood pressure and PWV.

The public health implications in this study is minimal considering the pilot nature of the studies. However, the study has provided the basis for the condition of more detailed studies, based on some interesting findings observed in this study. The next step is to investigate the effects of serum 25(OH)D concentration on EF and whole body NO production in RCTs or in prospective studies. Longer duration of studies, using various doses of vitamin D, involving various population and age groups, using different techniques of EF measurements are the criteria that should be considered in planning the future studies. Furthermore, targeting

population groups with evidence of altered glucose homeostasis, e.g. those with diabetes may be particularly informative since my systematic review and meta-analysis suggested that vitamin D supplementation may be beneficial in this population group.

In addition, there is a scope for further *in vivo* and *in vitro* in human and animal studies which investigate the mechanism through which $1,25(OH)_2D_3$ may regulate EF. As $1,25(OH)_2D_3$ involved in the upregulation of VEGF and its receptors in the endothelial cells (Zhong *et al.*, 2014), an *in vivo* study on the mechanism of $1,25(OH)_2D_3$ in preventing hypoxia or oxidative stress condition might be worth explored in details. In addition, an animal study may be conducted to understand the mechanism of which $1,25(OH)_2D_3$ involved in the alteration of basal tone of the endothelium, which normalised blood pressure, cardiac systolic function and renin-aldosterone level as reported by (Zhou *et al.*, 2008). Such studies should focus on effects which are mediated by NO.

Appendix 2 1: Systematic review and meta-analysis published in the European Journal of Nutrition

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ORIGINAL CONTRIBUTION

Effects of vitamin D supplementation on endothelial function: a systematic review and meta-analysis of randomised clinical trials

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Abstract

Background In addition to regulating calcium homoeostasis and bone health, vitamin D influences vascular and metabolic processes including endothelial function (EF) and insulin signalling. This systematic review and meta-analysis of randomised clinical trials (RCTs) were conducted to investigate the effect of vitamin D supplementation on EF and to examine whether the effect size was modified by health status, study duration, dose, route of vitamin D administration, vitamin D status (baseline and post-intervention), body mass index (BMI), age and type of vitamin D.

Methods We searched the Medline, Embase, Cochrane Library and Scopus databases from inception until March 2015 for studies meeting the following criteria: (1) RCT with adult participants, (2) vitamin D administration alone, (3) studies that quantified EF using commonly applied methods including ultrasound, plethysmography, applanation tonometry and laser Doppler.

Results Sixteen articles reporting data for 1177 participants were included. Study duration ranged from 4 to 52 weeks. The effect of vitamin D on EF was not significant (SMD: 0.08, 95 % CI −0.06, 0.22, $p = 0.28$). Subgroup analysis showed a significant improvement of EF in diabetic subjects (SMD: 0.31, 95 % CI 0.05, 0.57, $p = 0.02$). A non-significant trend was found for diastolic blood pressure ($\beta = 0.02$; $p = 0.07$) and BMI ($\beta = 0.05$; $p = 0.06$).

Conclusions Vitamin D supplementation did not improve EF. The significant effect of vitamin D in diabetics and a tendency for an association with BMI may indicate a role of excess adiposity and insulin resistance in modulating the effects of vitamin D on vascular function. This remains to be tested in future studies.

Keywords Vitamin D · Nitric oxide · Flow-mediated dilation · Forearm blood flow · Pulse wave velocity · Cardiovascular risk

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Introduction

Cardiovascular diseases (CVDs) are a major public health concern and contribute to >30 % of overall mortality worldwide [1]. The pathogenesis of CVDs is multifactorial, and a critical step in the onset and advancement of CVDs is the formation of atherosclerotic lesions [2]. One of the earliest stages of the atherosclerosis process is the impairment of endothelial function (EF) [3].

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The pathophysiology of endothelial dysfunction is complex and involves multiple mechanisms including overproduction of reactive oxidative species, inflammatory cytokines and pro-atherogenic lipoproteins together with an imbalance between vasodilating and vasoconstricting molecules. Impairment of vasodilatation may be due to reduced bioavailability of nitric oxide (NO), which is produced by the endothelial cells and which is involved in multiple physiological processes including vasodilation, inflammation and platelet aggregation [4].

Vitamin D is a pro-hormone, which is mostly known for its involvement in the regulation of calcium homoeostasis and bone remodelling [5]. However, vitamin D is also essential for several non-musculoskeletal functions including regulation of vascular tone, gluco-insular homoeostasis and immunity [5]. Vitamin D receptors (VDRs) are expressed in several tissues notably endothelial cells, vascular smooth muscle cells and cardiomyocytes [6]. The active form of vitamin D (1α, 25-dihydroxyvitamin D_3, 1, $25(OH)_2D_3$) is a direct transcriptional regulator of endothelial NO synthase [7]. A recent study has shown that VDR mutant mice have lower NO bioavailability, leading to endothelial dysfunction, increased arterial stiffness, increased aortic impedance, structural remodelling of the aorta and impaired systolic and diastolic heart function [8]. However, observational studies evaluating the association of vitamin D with CVD risk have reported mixed results. A significant inverse relationship between low vitamin D status, as assessed by serum 25-hydroxy vitamin D (25(OH)D) and increased risk of major cardiovascular events and chronic diseases such as myocardial infarction (MI), stroke, hypertension and type 2 diabetes, has been reported [9–11], but this has not been confirmed in other cohorts [12, 13]. These discrepant results may be ascribed to the differences between study designs and phenotypic characteristics of study participants including (1) duration of follow-up, (2) cut-off values for the definition of deficient vitamin D status, (3) diagnostic criteria for the identification and classification of cardiovascular outcomes, (4) confounding factors (i.e. diet, sun exposure, seasonality and physical activity) and (5) health status of the participants in the cohorts [14]. Randomised clinical trials (RCTs) examining the effects of vitamin D supplementation on EF have also reported contradictory results; whilst some studies have reported improvement in EF [15–17] others have observed no effect of vitamin D supplementation [18–30]. A recent meta-analysis has showed a non-significant effect of vitamin D supplementation on changes in flow-mediated dilation measured by ultrasound after post-occlusion hyperaemia. The study showed that effects were greater in short studies (<16 weeks) and in subjects with raised systolic and diastolic blood pressure (BP) [31].

The method for the assessment of EF in humans depends on the availability of resources and equipment, technical and research expertise and, most importantly, by the research question under investigation. The most commonly used methods to measure dynamic vascular responses are as follows: (1) ultrasound to assess the increase in diameter of large arteries following post-occlusive hyperaemia, (2) plethysmography to assess changes in forearm blood flow during infusion of pharmacological agents targeting endothelial-related mechanisms (e.g. acetylcholine or sodium nitroprussiate) and (3) applanation tonometry by measuring pulse wave velocity (PWV) of peripheral arteries [32].

We aimed to conduct a systematic review and meta-analysis of RCTs investigating the effect of supplemental vitamin D on EF. The secondary aim of the study was to determine whether the effect size was modified by health status, study duration, dose, route of vitamin D administration, baseline vitamin D status and changes in 25(OH) D after supplementation, body mass index (BMI), age and type of vitamin D (vitamin D_2 or vitamin D_3).

Methods

The present systematic review was conducted according to the Cochrane guidelines [33], and it is reported according to PRISMA guidelines [34].

Literature search

Four databases (Medline, Embase, Scopus and Cochrane Library) were used to search for articles from inception until March 2015. In addition, a manual search of reference lists of relevant reviews and articles included in the systematic review was performed. The search was conducted based on pre-defined search terms [Ergocalciferol OR Cholecalciferol OR vitamin D OR Vitamin D_2 OR vitamin D_3 OR 25(OH)D] And [Endotheli* OR Endotheli* dysfunction OR FMD or Hyperaemia OR Plethysmography OR Flow mediated OR Endothelial-dependent OR Vasomotor or Vasoacti* OR Blood flow OR Brachial OR Vasodilat* OR Dilat* OR Vascular resistance OR Pulse Wave OR Augmentation index OR Arterial stiffness OR Digital volume pulse OR Pulse amplitude tonometry OR Arterial compliance].

Study selection

The following criteria were applied to identify articles to be included in this systematic review and meta-analysis: (1) RCTs (no further exclusion criteria were applied in relation to study design or blinding); (2) studies involving

adults aged 18 years or more, and no exclusion criteria were applied for health status, smoking history or body size; (3) vitamin D administered alone, i.e. not combined with other drugs or nutritional interventions; studies were not excluded on the basis of the dose, duration of follow-up, route of administration of vitamin D or type of administration (i.e. tablet, capsule, solution or as fortified food) and type of assay used for the determination of 25(OH)D concentrations; (4) studies reporting changes in EF measured by ultrasound, venous occlusion plethysmography, photoplethysmography, pulse wave velocity, pulse amplitude tonometry, laser Doppler flowmetry; (5) no language or time restrictions were applied in searching the databases.

Two investigators (AMH and MS) independently screened the titles and abstracts of the articles to evaluate eligibility for inclusion. If consensus was reached, articles were either excluded or moved to the next stage (full text). If consensus was not reached, the articles were moved to the full-text stage. The full texts of the selected articles were appraised critically to determine eligibility for inclusion in the systematic review. Disagreements were resolved by discussion among the authors until the consensus was reached.

Data extraction and quality assessment

The following information was extracted from the eligible articles: (1) authors, journal details and year of publication; (2) participants (total number, male/female ratio, age, health status); (3) study characteristics (country, design, inclusion/exclusion criteria, description of measurement protocols; (4) vitamin D intervention (type, formulation, dose, duration of follow-up, route of administration); (5) EF measurement (instrument, position, duration of cuffing); and (6) circulating concentrations of vitamin D before and after intervention.

In addition, we adopted the modified Jadad score to assess the risk of bias of the included studies; possible scores ranged from 0 to 5, and a score of ≤ 3 indicates high risk, while a score of >3 indicates low risk of bias [35].

Statistical analysis

Serum concentrations of 25(OH)D given in ng/mL were converted to nmol/L (1 ng/mL = 2.496 nmol/L) [36]. Several methods were used to assess EF in humans including flow-mediated dilation (FMD), forearm blood flow (FBF), pulse wave analysis (PWA) and laser Doppler (LD) with the results obtained from these methods reported on different scales. Therefore, to allow comparison of effect sizes between studies, standardised mean differences (SMDs) were used as a summary statistic. SMD is estimated from the difference between the mean outcome values of the intervention and control groups divided by the pooled standard deviation (SD) of the outcome values; this converts the estimated effect to SD units. SMD of 0.2, 0.5 and 0.8 represents small, medium and large effect sizes, respectively [37]. In addition, different methods were frequently used in the same trial to assess EF, as given in Table 1, and therefore, this lack of independence of the EF measurement in each trial was taken into consideration in the derivation of the pooled effect size. Statistical analyses were performed by using Comprehensive Meta-Analysis software (version 2, Biostat, Englewood, NJ, USA). Data synthesis, including calculation of effect sizes with 95 % confidence intervals, was accomplished by employing a random-effect model using inverse variance weighting. Forest plots were generated for graphical presentation of the effect of supplemental vitamin D on EF. For this purpose, the mean and SD of the EF measure before and after the intervention period (for both vitamin D intervention and control) were extracted and used in the analysis. For studies that reported changes in EF at two or more time-points (e.g. acute and chronic effects of vitamin D supplementation), the last EF measurement was used in the meta-analysis. Data not provided in the main text or tables were extracted from the figures.

Subgroup analyses were undertaken to investigate the variables that may have influenced the effects of supplementation on EF. These factors included: health status, type (vitamin D_2 or D_3) and the frequency of administration (single dose, daily–weekly or monthly) of vitamin D supplementation. Random-effect meta-regression analyses were used to determine whether participant baseline characteristics (age, BMI, systolic and diastolic blood pressure, baseline concentration of 25(OH)D) influence the effect of vitamin D supplementation (vitamin D_2 or D_3) on EF. Furthermore, meta-regression analyses were conducted to investigate the influence of other factors including vitamin D dose, baseline 25(OH)D, change in 25(OH)D concentration after supplementation, duration of interventions, sample size and quality score (Jadad score) on the effect of vitamin D supplementation on EF.

Heterogeneity between studies was evaluated using Cochrane Q statistics; $p > 0.1$ indicates significant heterogeneity. The I^2 test was also used to evaluate consistency between studies where a value <25 % indicates low risk of heterogeneity, 25–75 % indicates moderate risk of heterogeneity and >75 % indicates high risk of heterogeneity [38]. The evidence of publication bias was assessed by visual inspection of the funnel plots and by the Egger's regression test [39].

Results

Search results

The process of screening and selection of studies is summarised in Figure S1 of the online supplementary

Table 1 Summary of findings

Author	Country	Compliance	Health status	Outcome	Sample size	Male (*N*)	Age (years)	BMI (kg/m^2)
Breslavsky et al. (2013) [15]	Israel	Not reported	T2D	AI	47	22	67	29
Gepner et al. (2012) [18]	US	Not reported	Healthy	PWV, FMD, AI	109	0	64	26
Harris et al. (2011) [16]	US	Not reported	Healthy OW	FMD	45	21	29	30
Hewitt et al. (2013) [19]	Australia	100 % compliance	CKD	PWV, FMD	60	29	60	29
Larsen et al. (2012) [20]	Denmark	99 % compliance	Ht (CVD)	AI, PWV	130	35	60	28
Longenecker et al. (2012) [21]	US	99 % compliance	HIV	FMD	45	35	47	27
Marckmann et al. (2012) [22]	Denmark	100 % compliance	CKD	PWV, FMD	52	39	71	25
Sokol et al. (2012) [23]	US	99 % compliance	CHD	RH-PAT	90	66	55	30
Stricker et al. (2012) [24]	Switzerland	100 % compliance	PAD (CVD)	AI	62	38	72.9	27
Sugden et al. (2008) [17]	UK	100 % compliance	T2D	FMD	34	18	65	31
Witham et al. (2010) [25]	UK	100 % compliance	T2D	FMD	61	41	G1:65 G2:63*	G1:31 G2:32
Witham et al. (2012) [26]	UK	100 % compliance	Stroke (CVD)	FMD	58	42	66	27
Witham et al. (2013) [27]	UK-South Asian	100 % compliance	Healthy	FMD, PWV, AI, LD-ION	50	0	41	27
Witham et al. (2013) [28]	UK	100 % compliance	MI (CVD)	RHI	75	52	64	27
Witham et al. (2013) [29]	UK	99 % compliance	ISH (CVD)	FMD, PWV	159	82	77	28
Yiu et al. (2012) [30]	China	Not reported	T2D	FMD, PWV	100	66	50	25

Author	SBP/DBP (mmHg)	Vit D dose (IU)	Duration (frequency)	Formulation (route)	Baseline 25(OH)D (assay)	Assay name, company	Δ 25(OH)D	Vit D/day (IU)	Jadad score
Breslavsky et al. (2013) [15]	153/74	1000 (D_3)	52 w (D)	Capsule (oral)	29 (IA)	Not stated	17	1000	3
Gepner et al. (2012) [18]	122/72	2500 (D_3)	16 w (D)	Biscuits (oral)	78 (LC–MS)	Not stated	39	2500	5
Harris et al. (2011) [16]	123/74	60,000 (D_3)	16 w (M)	Capsule (oral)	36 (IA)	Immunodiagnostic systems, Fountain Hills, AZ	66	2000	4
Hewitt et al. (2013) [19]	131/76	50,000 (D_3)	8 w (WK)	Solution (oral)	42 (IA)	DiaSorin Inc, Stillwater, MN	42	7142	4
Larsen et al. (2012) [20]	131/77	3000 (D_3)	20 w (D)	Tablet (oral)	57 (IA)	Liaison; DiaSorin, Saluggia, Italy	52	3000	5
Longenecker et al. (2012) [21]	118/80	4000 (D_3)	12 w (D)	Capsule (oral)	19 (IA)	Immunodiagnostic Systems, Fountain Hills, AZ, USA	12	4000	5
Marckmann et al. (2012) [22]	135/72	40,000 (D_3)	8 w (WK)	Capsule (oral)	28 (LC–MS)	(LCMSMS 1, Applied Biosystems, Dionex, Sunnyvale, California, US	118	5714	4
Sokol et al. (2012) [23]	133/76	50,000 (D_2)	12 w (WK)	Tablet (oral)	84 (LC–MS)	Quest Diagnostics, Teterboro, NJ, USA	67	7142	3
Stricker et al. (2012) [24]	136/74	100,000 (D_3)	4 w (SD)	Solution (oral)	41 (IA)	DiaSorin, Saluggia, Italy	19	3571	4
Sugden et al. (2008) [17]	141/80	100,000 (D_2)	8 w (SD)	Solution (oral)	38 (IA)	I.D.S., Tyne and Wear, UK	23	1785	3

Table 1 continued

Author	SBP/DBP (mmHg)	Vit D dose (IU)	Duration (frequency)	Formulation (route)	Baseline 25(OH)D (assay)	Assay name, company	Δ 25(OH)D	Vit D/day (IU)	Jadad score
Witham et al. (2010) [25]	G1:141/76 G2:128/72	G1:100,000 (D_3) G2:200,000 (D_3)	16 w (SD)	Solution (oral)	G1:46 G2:43 (IA)	IDS, Boldon, UK	28 18	G1:892 G2:1785	5
Witham et al. (2012) [26]	129/72	100,000 (D_2)	16 w (SD)	Solution (oral)	38 (IA)	DiaSorin Ltd, Bracknell, UK	12	892	3
Witham et al. (2013) [27]	121/78	100,000 (D_3)	8 w (SD)	Solution (oral)	27 (IA)	IDS Ltd UK	10	1785	5
Witham et al. (2013) [28]	128/72	100,000 (D_3)	24 w (2 M)	Solution (oral)	47 (IA)	I.D.S, Bachem UK, Merseyside, UK	13	1785	4
Witham et al. (2013) [29]	163/78	100,000 (D_3)	52 w (3 M)	Solution (oral)	45 (IA)	Not stated	25	1190	5
Yiu et al. (2012) [30]	146/81	5000 (D_3)	12 w (D)	Tablet (oral)	54 (IA)	I.D.S, (company not stated)	92	5000	3

Study designs for all of the studies are parallel, double-blind, placebo-controlled randomized trial. *I.D.S* immunodiagnostic system

N number of subjects, *OW* overweight, *IR* insulin resistance, *AI* augmentation index, *PWV* pulse wave velocity, *FMD* flow-mediated dilatation, *RH-PAT* reactive hyperaemia peripheral arterial tonometry, *RHI* reactive hyperaemia index, *LD-ION* Laser Doppler iontophoresis, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *BMI* body mass index, *EF* endothelial function, * Different doses of vitamin D, *Group 1* 100,000 IU, *Group 2* 200,000 IU, *w* weeks, *D* daily, *WK* weekly, *M* monthly, *SD* single dose, *2M* every 2 months, *3M* every 3 months, Δ changes in vitamin D concentrations after supplementation. Vitamin D concentrations are reported in nmol/L. *IA* immunoassay, *LC–MS* liquid chromatography mass spectrometry, *CVD* cardiovascular disease group, *US* Unites States, *UK* United Kingdom, *Ht* hypertension, *MI* myocardial infraction, *ISH* isolated systolic hypertension, *CHD* coronary heart disease, *CKD* chronic kidney disease, *PAD* peripheral arterial disease, *T2D* type 2 diabetes, *HIV* human immunodeficiency virus

material. The primary search of the four databases produced 4159 articles after removal of duplicates. After title and abstract screening, 22 full-text papers were retrieved for further evaluation. Additionally, one study was found by manual searching references of the relevant reviews and studies. Examination of the full text of 23 articles yielded 16 studies, which were eligible to be included in this systematic review and meta-analysis. One trial [25] included two independent arms supplementing different vitamin D doses, which resulted in 17 independent interventions entered in the final meta-analysis.

Studies characteristics

The total number of participants from the 16 studies included in this systematic review was 1177 (607 females; 570 males) with median of 73 (range 34–159) participants per study. The median age was 63.2 (range 30–77) years. All RCTs included in the meta-analysis were parallel, double-blind, placebo-controlled trials. The duration of the trials ranged from 4 to 52 weeks (Table 1).

Three studies investigated the effect of vitamin D in healthy participants [16, 18, 40], two studies were conducted in patients with chronic kidney disease (CKD) [19, 22], four studies in diabetics [15, 17, 25, 30], six studies in patients with CVDs [20, 23, 24, 26, 28, 29] and one study in patients with HIV [21]. All trials supplemented vitamin D orally. Trials, however, utilised different forms of supplementation including tablets [20, 23, 30], solution [17, 19, 24–26, 28, 40], capsules [15, 16, 21, 22] and fortified biscuits [18]. The majority of the trials utilised vitamin D_3 with daily doses varying from 1000 IU/day [15] to 5000 IU/day [30].

Several methods were used to assess EF in the included trials. The most commonly used methods were as follows: FMD [16–19, 21, 22, 25, 29, 30], PWV [18–20, 22, 29, 30, 40] and augmentation index (AIx) [15, 18, 20, 24]. Other methods include laser Doppler flowmetry [40] and digital volume pulse [28] (Table 1).

Qualitative analysis

Three of the studies included in the present systematic review reported a significant improvement in EF in response to vitamin D administration [15, 17, 41], whereas the other 13 studies reported no effect of supplementation [18–30]. Ten studies described the methods of randomisation [18–23, 25, 27, 28, 30], and five studies stated the methods of allocation concealment [20, 21, 25, 27, 28]. The drug history of the participants was reported by all except three studies [15, 16, 27]. With the exception of two studies [16, 19], all other studies reported, and described, participant dropout. The quality of the included studies ranged from 3 to 5 (Jadad score), and eleven studies had a low risk of bias (Jadad score ≥4) (Table 1).

Meta-analysis

Meta-analysis of the 16 studies (1177 participants) showed that, overall, vitamin D supplementation did not improve EF (SMD: 0.08, 95 % CI −0.06, 0.22, $p = 0.28$) (Fig. 1). The effect of supplemental vitamin D on post-occlusive vasodilation of the brachial artery was not significant (FMD %, $N = 10$, +0.27 %, 95 % CI −0.36, 0.91, $p = 0.39$, Table S1, Online Supplementary Material). Heterogeneity between studies was not significant ($Q = 21.7$, $I^2 = 26.4$ %, $p = 0.15$). Subgroup analysis showed that vitamin D supplementation improved EF significantly in participants with type 2 diabetes ($N = 5$, SMD: 0.31, 95 % CI −0.05, 0.57, $p = 0.02$) (Table 2). This was confirmed by the significant effect of vitamin D supplementation in type 2 diabetic on changes in FMD % ($N = 4$, +0.81 %, 95 % CI 0.005, 1.61, $p = 0.04$, Table S1, Online Supplementary Material). The response of EF to vitamin D supplementation was not significantly modified by type of vitamin D, method of administration, baseline 25(OH)D concentrations or baseline health status of the participants (Table 2). Meta-regression analyses demonstrated a weak, positive effect of BMI (β: 0.05, SE: 0.02, $p = 0.06$) and of baseline diastolic blood pressure (β: 0.02, SE: 0.01, $p = 0.07$) in modifying the effect of vitamin D supplementation on EF (Table 3). BMI did not modify the association between type 2 diabetes and EF ($N = 6$, β: 0.04, SE: 0.04, $p = 0.23$), whereas lower baseline 25(OH)D concentrations were associated with a greater effect size in type 2 diabetic participants ($N = 6$, β: −0.02, SE: 0.01, $p = 0.03$) (Figure S3, Online Supplementary Material). The dose of vitamin D was not associated with significant changes in EF (Table 3 and Figure S4, Online Supplementary Material).

Publication bias

Visual inspection of the funnel plot showed modest evidence of asymmetric distribution of the effect size (Figure S2 of the online supplementary material), which was confirmed formally by the lack of significance of the Egger's test ($p = 0.08$).

Discussion

Overall, our meta-analysis demonstrated no effect of vitamin D supplementation on EF. In addition, baseline vitamin D and change in vitamin D concentration after supplementation were not associated with effects of vitamin D supplementation on EF. However, vitamin D supplementation resulted in a significant improvement in EF in patients with diabetes, and there was a positive trend towards greater effects of vitamin D on EF with increasing baseline BMI and diastolic blood pressure.

Several putative mechanisms could explain the positive effects of vitamin D on EF in some population groups, particularly in those at higher cardiovascular risk. Vitamin D is involved in the regulation of endothelial cell-dependent

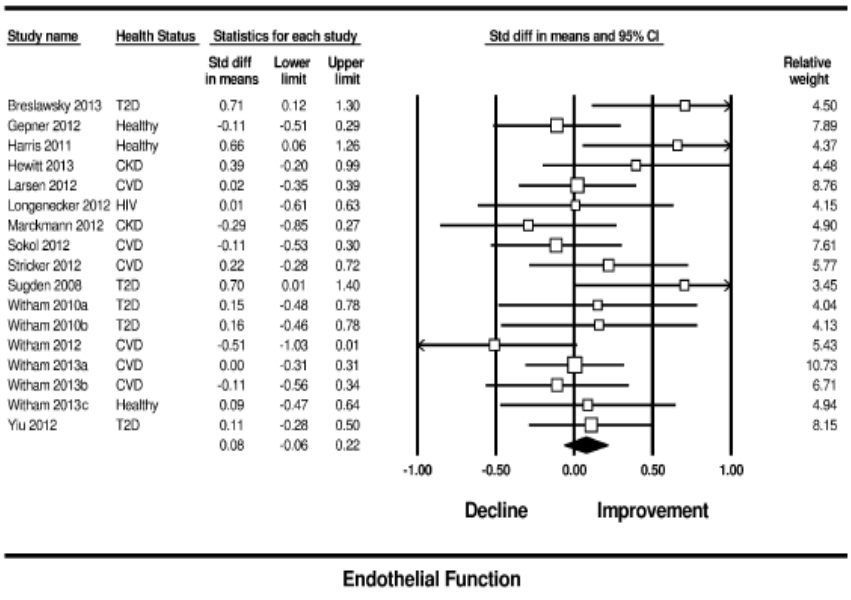

Fig. 1 Forest plot showing the effect of vitamin D supplementation on endothelial function. *T2D* type 2 diabetes, *CVD* cardiovascular disease, *CKD* chronic kidney disease. Relative weight for a random model allows for small size studies contributing in a similar magnitude to the pooled estimate. The marker may vary in size according to the weights assigned to the different studies. The pooled effect is represented using a diamond

Table 2 Sensitivity analysis to evaluate the influence of health status, administration of vitamin D and type of vitamin D dose on the effect of vitamin D supplementation on endothelial function

Group	No. of trials or subgroup	Effect size	95 % CI	*p*	*p* between groups
Health status					
Healthy	3	0.15	−0.28 0.59	0.47	0.23
HIV	1	0.009	−0.61 0.62	0.97	
Diabetes	5	0.31	0.05 0.57	0.02	
CKD	2	0.04	−0.62 0.71	0.89	
CVD	6	−0.05	−0.22 0.11	0.51	
Frequency of dose administration					
1–3 month	4	0.17	−0.14 0.48	0.29	0.71
Daily–weekly	7	0.02	−0.17 0.21	0.82	
Single dose	6	0.09	−0.20 0.40	0.53	
Baseline 25(OH)D concentration					
Normal (≥50 nmol/L)	4	−0.01	−0.21 0.17	0.84	0.27
Deficient (<50 nmol/L)	13	0.13	−0.06 0.32	0.17	
Vitamin D type					
D_2	3	−0.02	−0.61 0.58	0.95	0.72
D_3	14	0.09	−0.03 0.22	0.15	

T2D type 2 diabetes, *CVD* cardiovascular disease, *CKD* chronic kidney disease, D_2 ergocalciferol, D_3 cholecalciferol, *25(OH)D* 25 hydroxy vitamin D

Table 3 Meta-regression analysis to evaluate the association of potential modifiers of the effects of vitamin D supplementation on endothelial function

Covariates	Slope	SE	Q ($df = 1$)	*p* value
Baseline systolic BP (mmHg)	0.002	0.003	0.60	0.43
Baseline diastolic BP (mmHg)	0.02	0.01	3.1	0.07
Serum 25(OH)D at baseline (nmol/L)	−0.003	0.002	2.47	0.11
Change in serum 25(OH)D after supplementation (nmol/L)	−0.001	0.001	0.77	0.37
Study duration (weeks)	0.001	0.003	0.16	0.68
Vitamin D dose (IU)	−0.0001	0.00001	0.12	0.71
Age (years)	−0.003	0.004	0.95	0.32
BMI (kg/m^2)	0.05	0.02	3.50	0.06
Study sample size (*N*)	−0.001	0.001	1.43	0.23
Jadad score	−0.02	0.05	0.28	0.59

BP blood pressure, *BMI* body mass index, *N* number of study participants, *25(OH)D* 25 hydroxy vitamin D

vasodilation, which may be mediated by the effect of vitamin D metabolites on the renin–angiotensin–aldosterone system, a hormonal system that regulates blood pressure and fluid balance. A low plasma 25(OH)D predisposes to up-regulation of the renin–angiotensin system, smooth muscle proliferation and favours a pro-inflammatory state, which can increase the risk of hypertension and left ventricle hypertrophy [42]. The improvement in EF through vitamin D supplementation could also be mediated by the local effects of vitamin D metabolites on calcium metabolism in vascular smooth muscle cells and on the release of inflammatory cytokines, which may affect vascular contractility [43]. Vascular smooth muscle and endothelial cells express VDR as well as 1α-hydroxylase [44], allowing for autocrine production of 1, 25(OH)2D, which may act at the local level to modulate the effects of inflammatory cytokines on the vasculature, such as decreasing endothelial adhesion molecules, increasing NO production [45] and reducing platelet aggregation [46]. The activation of VDRs induces the transcription of a wide range of genes including those coding for vascular endothelial growth factor, which in turn promotes NO synthesis by endothelial cells. In addition, 1, 25$(OH)_2D_3$ is a direct regulator of endothelial NO synthase [8].

Vitamin D may also have beneficial effects on cardiometabolic health in those with hypertension [47–50], type 2 diabetes [11, 30, 51] and cardiovascular disease [52–54]. A meta-analysis of data from 21 prospective studied showed

an inverse association between vitamin D status and risk of type 2 diabetes [55]. In addition, cardiovascular disease is the main cause of premature mortality and morbidity in patients with CKD [22]. These cardiovascular complications may be related to hypovitaminosis D [56], which may be linked to the inability of renal mass to convert 25(OH)D to the active form of vitamin D, 1, 25-dihydroxyvitamin D [57]. However, our results did not show a significant effect of vitamin D supplementation on EF in patients with CKD, which could be explained by several factors including the small number of studies (only two trials), the short duration (8 weeks), the inadequacy of the vitamin D dose or the advanced stage of endothelial dysfunction.

In the present meta-analysis, we observed that vitamin D supplementation produced a significant improvement in endothelial function in individuals with type 2 diabetes. While the small number of trials included in the analyses ($N = 4$) calls for a cautious and objective interpretation of the results, we believe that they are supported by a robust mechanistic rationale and provide important insights into future studies. This apparent diabetes-specific effect may be explained by several mechanisms including the link between low 25(OH)D concentrations and (1) deterioration of β-cell function, (2) dysregulation of peripheral insulin signalling and (3) altered glucose disposal which are typically involved in the pathogenesis of type 2 diabetes [11, 14, 58]. These effects appear to be supported by the greater effect of vitamin D supplementation on EF in type 2 diabetic patients with insufficient vitamin D status. Vitamin D receptors and 1-α-hydroxylase are expressed in pancreatic β-cells, and therefore, an involvement in the regulation of insulin secretion may be expected [51]. In turn, 1, 25$(OH)_2$D activates transcription of the human insulin receptor gene, stimulates expression of the insulin receptor [59] and enhances insulin-mediated glucose transport in vitro [60]. In addition, insulin secretion is a calcium-dependent process and vitamin D metabolites have been linked to the regulation β-cell calcium pools, which promote insulin release [61]. The putative beneficial effects of vitamin D metabolites on EF may also be explained by the mechanistic interconnection between the insulin and NO pathways. The activation of the insulin receptor on the endothelial cells instead induces a vasodilatory response via the activation of the phosphoinositol-3-phosphate—Akt pathway which increases NO production by the enzyme endothelial nitric oxide synthase [62].

Our meta-regression analysis showed a trend for a greater improvement of EF in response to vitamin D supplementation in participants with high BMI. Growing evidence has shown that there is an inverse association between plasma 25(OH)D concentrations and BMI [63, 64]. Decreased bioavailability of vitamin D was found in obese subjects [63–65], which may be explained by adipose tissue sequestration and/or volumetric dilution of 25(OH)D [66], and may explain the tendency towards a greater effect of supplemental vitamin D on EF in subjects with greater adiposity. In addition, obesity and excess visceral adiposity are closely associated with insulin resistance and development of type 2 diabetes, which may explain the almost significant effect of vitamin D supplementation on EF in obese subjects. This may indirectly suggest that the magnitude of the effect size of vitamin D on EF may be correlated with the degree of metabolic derangement of the insulin signalling pathway.

Results may have been affected by the choice of the method used to measure vitamin D concentrations. Unlike chromatographic methods, immunoassays do not measure vitamin D_3 and vitamin D_2 independently, and this is a well-recognised limitation of immunoassays. The importance of being able to quantify both metabolites of vitamin D independently is becoming increasingly important in recent years with the evidence that vitamin D_3 is more biologically active than vitamin D_2 [67] as well as emerging evidence that 25$(OH)D_2$ concentrations are in the range of 1.5–10.0 nmol/l in several RCT and population-based studies, this contributing significantly to total 25(OH)D [68]. It is also important to point out that results of 25(OH)D using chromatographic methods show significant variation, mainly due to extraction and calibration problems associated with these methods. Such assay variation reinforces the need for all users of vitamin D assays to have appropriate QC and standardisation protocols in place.

Our meta-analysis has some limitations. First, the available trials had relatively small sample sizes with samples sizes of <100 in about 75 % of the trials included in the meta-analysis. Second, the variability in duration, dose and type of vitamin D supplementation, the different methods used to assess EF and the diversity in participant characteristics (age, sex and health status) may have introduced significant heterogeneity and have militated against observation of overall effects of vitamin D supplementation on EF in our meta-analysis. Third, not all studies adjusted for potential confounding factors that may have influenced the effect of vitamin D on EF such as sun exposure, seasonality, physical activity or dietary patterns. Finally, most of the study participants were aged between 40 and 77 years old, thus limiting the applicability of the findings to other life stages. Finally, studies have used different assays to measure 25(OH)D concentrations (Immunoassay, $N = 13$; Liquid Chromatography Mass Spectrometry, $N = 3$), which may have introduced a measurement bias. However, the exclusion of the three studies using LC–MS from the analysis did not modify the results, which provides support to the importance of vitamin D status in influencing the efficacy of vitamin D supplementation on vascular outcomes (data not shown).

We believe that the current evidence base is inadequate to draw firm conclusions about the protective role of supplemental vitamin D on EF and as a pharmaco-nutritional strategy for CVD prevention. However, our study provides important information on the effects of vitamin D supplementation on EF and shows that benefit may be anticipated in diabetics. This may indicate a potential role of insulin resistance in modulating the effects of vitamin D on vascular function. This hypothesis remains to be tested in future studies.

Acknowledgments The systematic review was conceived by JCM, TH and MS. AM, AA and MS searched, collected and analysed the data, and all authors contributed to data interpretation. AM, AA, IS and MS drafted the manuscript. All authors contributed to the final version of the manuscript. The corresponding author (AM) is the guarantor for the manuscript and had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final version of the paper.

Compliance with ethical standards

Conflict of interest None to Declare.

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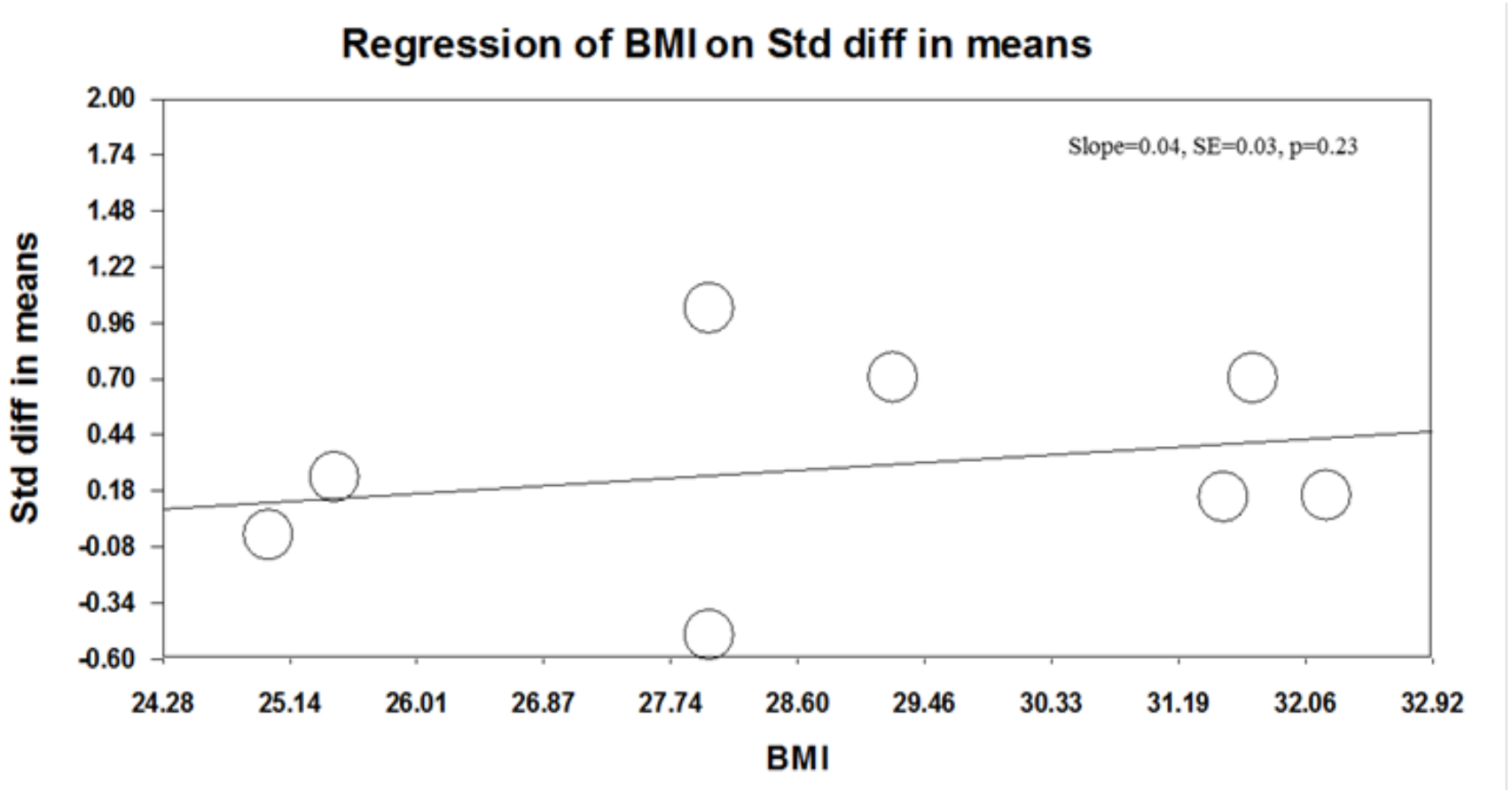
Appendix 2. 2: BMI did not modify the association between type 2 diabetes and EF



Appendix 2 3: Studies in the review with different assays to measure 25-OHD concentrations

Author	Compliance	Assay name, company	Vit D/day (IU)
Breslavsky et al. 2013 (Breslavsky *et al.*, 2013)	Not reported	Not stated	1000
Gepner et al. 2012 (Gepner *et al.*, 2012)	Not reported	Not stated	2500
Harris et al. 2011 (Harris *et al.*, 2011a)	Not reported	Immunodiagnostic systems, Fountain Hills, AZ	2000
Hewitt et al. 2013 (Hewitt *et al.*, 2013)	100% compliance	DiaSorin Inc, Stillwater, MN	7142
Larsen et al. 2012 (Larsen *et al.*, 2012)	99% compliance	Liaison; DiaSorin, Saluggia, Italy	3000
Longenecker et al. 2012(Longenecker *et al.*, 2012)	99% compliance	Immunodiagnostic Systems, Fountain Hills, AZ, USA	4000
Marckmann et al. 2012 (Marckmann *et al.*, 2012)	100% compliance	LCMSMS 1, Applied Biosystems, Dionex, Sunnyvale, California, US	5714
Sokol et al. 2012 (Sokol *et al.*, 2012)	99% compliance	Quest Diagnostics, Teterboro, NJ, USA	7142
Stricker et al. 2012 (Stricker *et al.*, 2012)	100% compliance	DiaSorin, Saluggia, Italy	3571
Sugden et al. 2008 (Sugden *et al.*, 2008)	100% compliance	IDS, Tyne & Wear, UK	1785
Witham et al. 2010 (Witham *et al.*, 2010)	100% compliance	IDS, Boldon, UK	G1:892 G2:1785
Witham et al. 2012 (Witham *et al.*, 2012)	100% compliance	DiaSorin Ltd, Bracknell, UK	892
Witham et al. 2013 (Beveridge and Witham, 2013)	100% compliance	IDS Ltd UK	1785
Witham et al. 2013 (Witham *et al.*, 2013b)	100% compliance	IDS, Bachem UK, Merseyside, UK	1785
Witham et al.2013 (Witham *et al.*, 2013c)	99% compliance	Not stated	1190
Yiu et al. 2012 (Yiu *et al.*, 2013)	Not reported	I.D.S, (company not stated)	5000
Dalan et al. 2016 (Dalan *et al.*, 2016a)	94% compliance	Electro-chemiluminescence immunoassay (ECLIA)	4000
Pilz et al 2015 (Pilz *et al.*, 2015)	Not reported	Chemiluminescence assay (IDS-iSYS 25-hydroxyvitamin D assay; Immunodiagnostic Systems Ltd., Boldon, UK)	2800
Witham et al 2015 (Witham *et al.*, 2015)	100%	Immunodiagnostic systems (IDS) and radioimmunoassay (RIA)	1666
Borgi et al 2016 (Borgi *et al.*, 2017)	Not reported	Radioimmunoassay (RIA), Diasorin Corporation	7142

Appendix 3. 1: Performing ADMA analysis

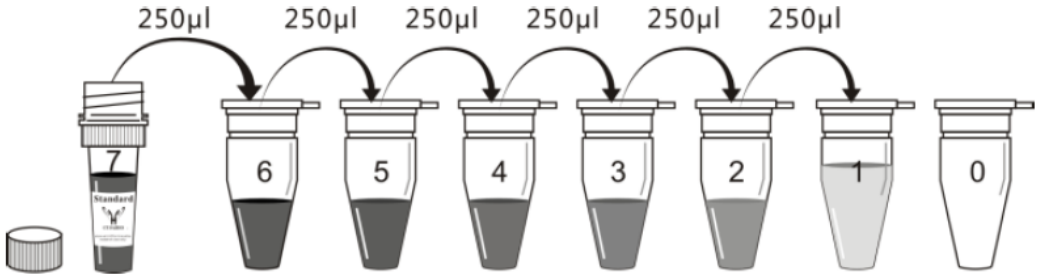
The measurement of ADMA in this PhD project was done using a commercial kit (CUSABIO ELISA kit CSB-E09298h). The following components were required to perform the analyses:1) Standard; 2) Biotin antibody; 3) HRP-avidin; 4) Biotin antibody Diluent; 5) HRP avidin diluent; 6) Sample diluent; 7) Wash buffer 8) TMB substrate and 9) Stop solution.

Reagent preparation

The plasma samples were collected in EDTA vacutainer tube. All reagents were brought to the room temperature (18 – 25ºc). The reagents, samples and standards were prepared as instructed;

1) Fresh standard was prepared for wash assay and used within 4 hours after the preparation.
2) Biotin antibody was centrifuged before opening. Biotin antibody requires 100-fold dilution (10 µl of Biotin-antibody + 990 µl of Biotin-antibody diluent).
3) HRP –avidin was centrifuged before opening. HRP –avidin requires a 100-fold dilution ((10 µl of HRP –avidin + 990 µl of HRP –avidin diluent).
4) Twenty ml of Wash Buffer was diluted into deionized water to prepare 500 ml of wash buffer.
5) Standard vial was centrifuged at 6000-10000rpm for 30s. Standard was then reconstituted with 1.0 ml of sample diluent. This reconstitution produced a stocl solution of 500 ng/ml.

   250 µl of sample diluent were pipetted into each tube (S0-S6) **(Figure 3.1A)**. The stock solution was used to produces a 2-fold dilution series. Each tube was mixed thoroughly before the next transfer. The undiluted standard served as the high standard (500ng/ml). Sample diluent served as the zero standard (0ng/ml)



Tube	S7	S6	S5	S4	S3	S2	S1	S0
ng/ml	500	250	125	62.5	31.2	15.6	7.8	0

Figure 3.1A: The dilution procedures for ADMA standard

Assay procedure

In this procedure, all samples and standards were assayed in duplicate. A plate map was used to record the location of standards and samples.

The following steps were followed to perform the assay:

1) 100 µL of standard and sample were added per well. The plate was covered with adhesive strip and incubated for 2 hours at 37ºC.
2) The liquid in each well were then removed, but not washed.
3) 100 µL of Biotin-antibody were added to each well. The plate was covered with a new adhesive strip and incubated for 1 hour at 37ºC.
4) Each well was then aspirated and washed. This process was repeated for two times for a total of three washes. Each well was filled with wash buffer (200 µL) using manifold dispenser and let it stand for 2 minutes. After the last wash, any remaining wash buffer was removed by aspiring or decanting. The plate was inverted and blotted against a clean paper towels.
5) 100 µL of HRP-avidin was added to each well. The plate was covered with a new adhesive strip and incubated for 1 hour at 37ºC.
6) The aspiration/wash process in step 4 was repeated.
7) 90 µL of TMB Substrate was added to each well and incubated for 15 – 30 minutes at 37ºC and protected from light.

8) 50 µL of stop solution was added to each well and the plate was gently tapped to ensure thoroughly mixed.
9) The optical density in each well were determined within 5 minutes using a microplate reader set to 450 nm.

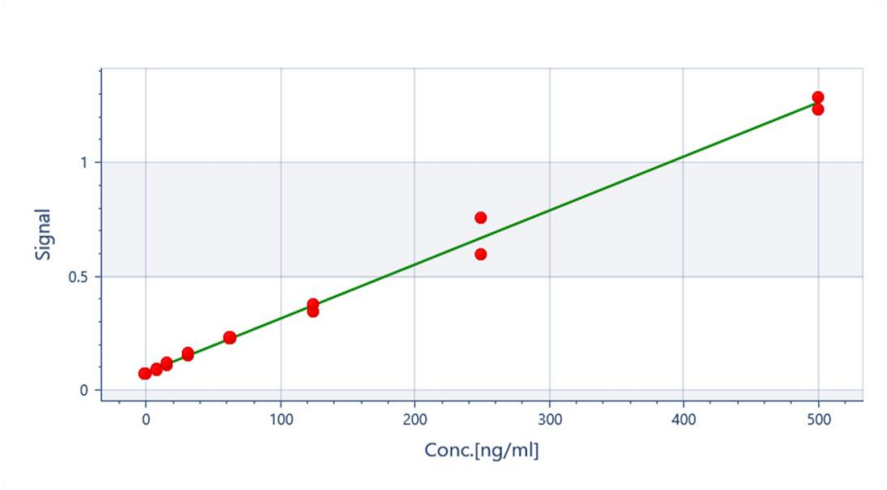


Figure 3.1B: Standard curve ADMA

Appendix 3. 2: Performing 3NT analysis

The measurement of 3-NT in this PhD project was done using a commercial kit (ABCAM ab113848). The following components were required to perform the analyses:1) 3NT BSA standard; 2) Extraction buffer; 3) Wash buffer; 4) Blocking solution; 5) HRP conjugated 3NT detector antibody and 6) Development solution

Reagent preparation

All reagents were brought to the room temperature (18 – 25ºc). The reagents, samples and standards were prepared as instructed;

1) Wash buffer was prepared by adding 25mL 20X Wash Buffer to 475 Nano pure water.
2) 2X Incubation Buffer were prepared by adding 10mL 10X Blocking Buffer to 40mL 1X Wash Buffer.
3) 2 X HRP-Detector antibodies were prepared immediately before use. 12µL 1000X Detector.
4) By pipetting, the standard was reconstituted with 1 mL 1X Wash Buffer. It was then allowed to sit for 10 minutes before the pipetting were repeated to ensure thorough reconstitution.

Standard preparation

To create a 3-fold standard curve, seven tubes were labelled with #1-7. 600 µL from stock was transferred to tube #1. 600 µL of 1X Wash buffer were then added to each of tube #2 through #7. 300 µL from stock was transferred from tube #1 to tube #2 and were mixed thoroughly. 300 µL was transferred from #2 to #3 using a fresh pipette tip and were mixed thoroughly. The procedures were repeated for tubes #4 through #7. 1X Wash buffer was used as the zero standard tube #8 **(Figure 3.2A)**.

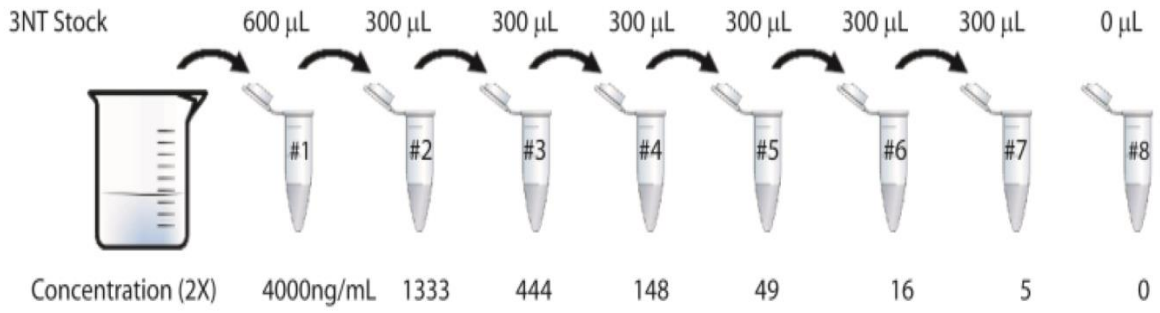


Figure 3.2A: Dilution procedures for Nitrotyrosine standard

Assay procedure

In this procedure, all samples and standards were assayed in duplicate. A plate map was used to record the location of standards and samples.

The following steps were followed to perform the assay:

1. 50 µL of each diluted standards was added to each of the sample wells in the plate.

2. 50 µL of each samples were pipetted into individual wells.

3. 50 µL of 2X HRP detector Antibody was pipetted into each well of the plate (for both standard and test samples)

4. The plate was covered/sealed and incubated for 2 hours in room temperature, on a plate shaker at 300rpm.

5. After the required incubation time, the plate was aspirated and washed for four times by using 300 µL 1X Wash Buffer into each well. The plate was inverted and blotted against clean paper towels to remove excess liquid.

6. 100 µL HRP development Solution was pipetted to each empty well and the blue colour development was immediately recorded with time in the microplate reader with the following settings:

Mode:	Kinetic
Wavelength:	600 nm
Time:	15 min
Interval:	20 sec – 1 min
Shaking:	Shake between readings

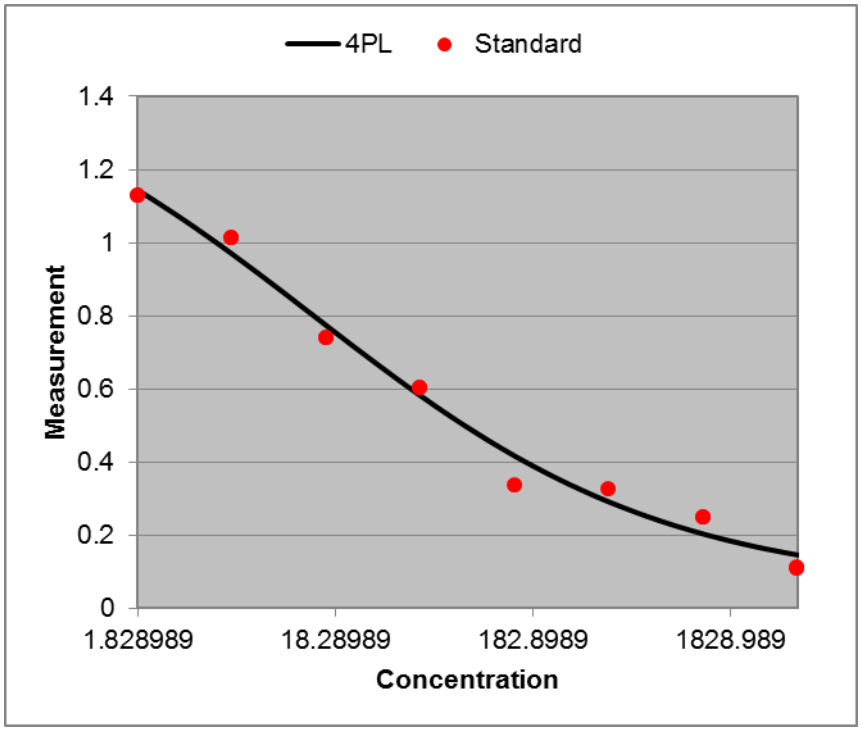


Figure 3.2B: Standard curve 3NT

Appendix 3. 3: Performing VEGF analysis

The measurement of VEGF in this PhD project was done using a commercial kit from R&D Systems™ Quantikine® ELISA to measure VEGF.

Reagent preparation

All reagents were brought to the room temperature (18 – 25ºc). The reagents, samples and standards were prepared as instructed;

Wash Buffer: 20mL of the Wash Buffer Concentrate were added to distilled water to prepare 500mL of Wash Buffer.

Substrate Solution: Colour Reagents A and B were mixed together in equal volumes within 15 minutes of use. 200 µL of the resultant mixture was required per well. This solution should be protected from light.

VEGF Standard: VEGF standard was reconstituted with Calibrator Diluent RD6U. This reconstitution produced a stock solution of 2000pg/mL.

Plasma samples: 500 µL of Calibrator Diluent RD6U were pipetted into each tube (six polypropylene tubes were required for this preparation). Stock solution was used to produce the dilution series. Each tube was mixed thoroughly before the next transfer. 200 pg/mL standard were served as the high standard **(Figure 3.6)**.

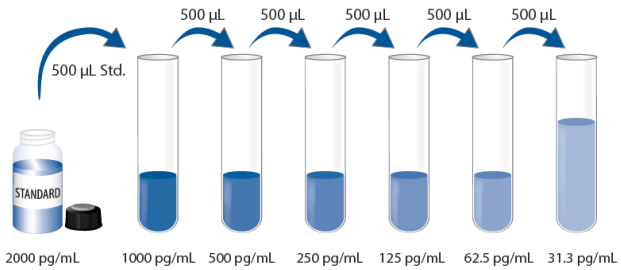


Figure 3.3A: Dilution procedures for VEGF standard

Assay procedure

In this procedure, all samples and standards were assayed in duplicate. A plate map was used to record the location of standards and samples.

The following steps were followed to perform the assay:

1. All reagents, working standards and samples were prepared as directed.
2. 100 µL of Assay Diluent RD1W were added to each well.
3. 100 µL of Standard, control and sample were added to each well. The plate was then covered with adhesive tape and were incubated for 2 hours at room temperature.
4. Each well was then being washed and aspirated. The process was repeated twice for a total of three washes.
5. 200 µL of VEGF Conjugate were added to each well and were incubated for 2 hours at room temperature.
6. The aspiration/wash were repeated.
7. 200 µL of Substrate Solution was added to each well. The well should be protected from light and incubated for 25 minutes at room temperature.
8. 50 µL of stop solution was added to each well.
9. The optical density in each well were determined within 30 minutes using a microplate reader set to 450 nm.

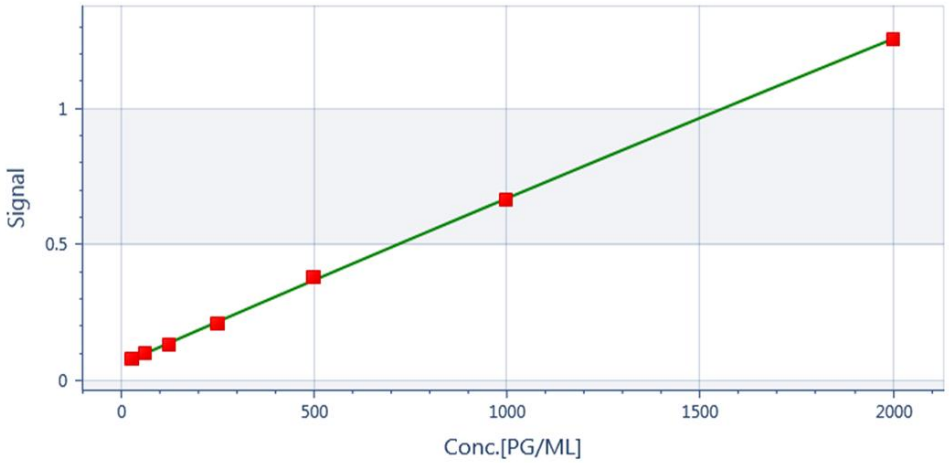


Figure 3.3B: Standard curve VEGF

Appendix 3. 4: Performing ET-1 analysis

The measurement of ET-1 in this PhD project was done using a commercial kit from Enzo Life Sciences, Inc (catalogue #ADI-900-020A).

Principle of the assay

Samples and standards were added to wells coated with a monoclonal antibody specific for ET-1. The plate was then incubated for 30 minutes. Then, the plate was washed, leaving only bound ET-1 on the plate. A solution of HRP labelled monoclonal antibody to ET-1 was added. This binds the ET-1 captured on the plate. The plate was incubated again for 30 minutes. Then, the plate was washed to remove excess HRP labelled antibody and TMB substrate solution was added. The substrate generates a blue colour when catalysed by the HRP. Lastly, stop solution was added to stop the substrate reaction and the resulting yellow colour were read at 450 nm.

Reagent preparation

The reagents, samples and standards were prepared as instructed;

Wash Buffer: Wash buffer was prepared by diluting 50 mL of Wash Buffer Concentrate with 950mL of deionized water.

ET-1 standards: Eight 12 x 75mm polypropylene were labelled with tubes #1 through #8. 450 µL of assay buffer were pipetted into tube #1. 250 µL of the assay buffer were pipetted into tubes #2 through #8. 50 µL of 1,000pg/mL standard stock were added into tube #1 and were vortex thoroughly. 250 µL of tube #1 were added to tube #2 and were vortex thoroughly. 250 µL of tube #2 were added to tube #3 and were vortex thoroughly. This procedure was continued for tubes #4 through #8 **(Figure 3.4A)**.

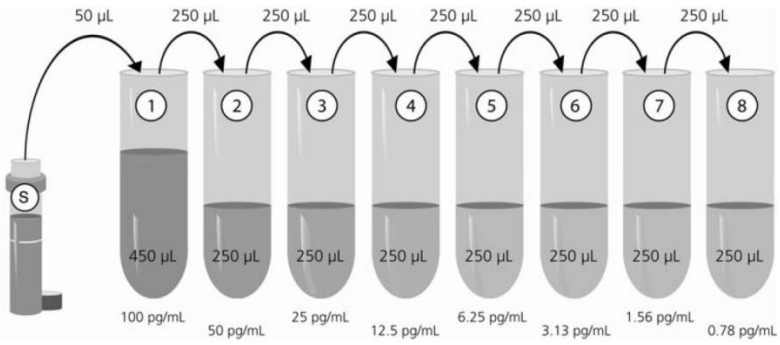


Figure 3.4A: Dilution procedures for ET-1 standard

ET-1 Antibody: The antibody was prepared by diluting 10 µL of the supplied antibody concentrate with 1 ml of antibody diluent.

Assay procedure

In this procedure, all samples and standards were assayed in duplicate. A plate map was used to record the location of standards and samples.

The following steps were followed to perform the assay:

1. 100 µL of assay buffer was pipetted into S0 (0pg/mL standard) wells.
2. 100 µL of standards #1 through #8 were pipetted to the bottom of the appropriate wells.
3. 100 µL of the samples were pipetted to the bottom of the appropriate wells.
4. The plate was sealed and incubated for 1 hour at room temperature.
5. After the incubation period, the contents of the wells were emptied and washed by adding 400 µL of wash buffer to each well. This procedure was repeated for 4 more times for a total of 5 washes. After the final wash, the wells were aspirated and the plate was tapped firmly on a paper towel to remove any remaining wash buffer.
6. 100 µL diluted antibody was pipetted into each well except the blank.
7. The plate was sealed and incubated for 30 minutes at room temperature.
8. Once again the plate was washed as in step 5.
9. 100 µL substrate was pipetted into each well.
10. The plate was incubated for 30 minutes at room temperature.
11. 100 µL of stop solution was pipetted into each well.
12. The optical densities in each well were determined using a microplate reader set to 450 nm.

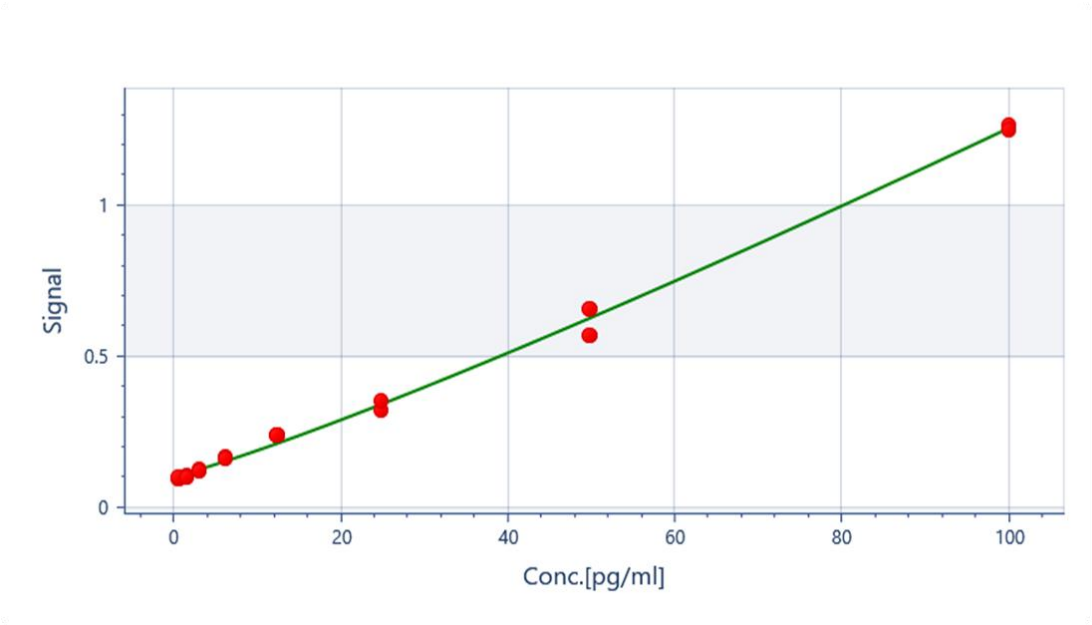


Figure 3.4B: Standard curve ET-1

Appendix 3. 5: Performing cGMP analysis

The measurement of cGMP in this PhD project was done using a commercial kit from Enzo Life Sciences, Inc (catalogue #ADI-900-013).

Reagent Preparation

cGMP Standard: 5,000 pmol/ml cGMP standard solution were brought to the room temperature (18 – 25ºc). Six 12 x 75 mm glass tubes #1 to #6 were labelled. 900 µL standard diluent (assay buffer) was pipetted into tube #1 and 800 µL of standard diluent into tubes #2-6. 100 µL of the 5,000 pmol/mL standard were added to tube #1 and were vortexed thoroughly. 200 µL of tube #1 were added to tube #2 and were vortexed thoroughly. These processes were continued for tubes #3 through #6.

Wash Buffer: The wash buffer was prepared by diluting 5ml of the supplied concentrate with 95ml of deionized water.

Assay procedure

In this procedure, all samples and standards were assayed in duplicate. A plate map was used to record the location of standards and samples.

1. 100 µL of standard diluent (assay buffer) was pipetted into the NSB and the Bo (0 pmol/mL standard) wells.
2. 100 µL of standards were pipetted into the appropriate wells.
3. 100 µL of samples were pipetted into the appropriate wells.
4. 50 µL of standard diluent were pipetted into the NSB wells.
5. 50 µL of blue conjugate were pipetted into each well, except the TA and Blank wells.
6. 50 µL of yellow Antibody were pipetted into each well, except the Blank, TA and NSB wells.
7. The plate was covered and incubated at room temperature for 2 hours on a plate shaker at ~500rpm.
8. After the incubation period, the contents were emptied and washed by adding 400 µL of wash solution to every well. This procedure was repeated for 2 times for a total of 3 washes.
9. After the final wash, the wells were aspirated and the plate was tapped firmly on a lint free paper towel to remove any remaining wash buffer.
10. 5 µL of blue conjugate was added to the TA wells.

11. 200 µL of the pNpp Substrate solution was added to every well and then incubated at room temperature for 1 hour without shaking.
12. 50 µL of stop solution was added to every well. This stopped the reaction and the plate were read immediately.
13. The optical density in each well were determined using a microplate reader set to 405 nm.

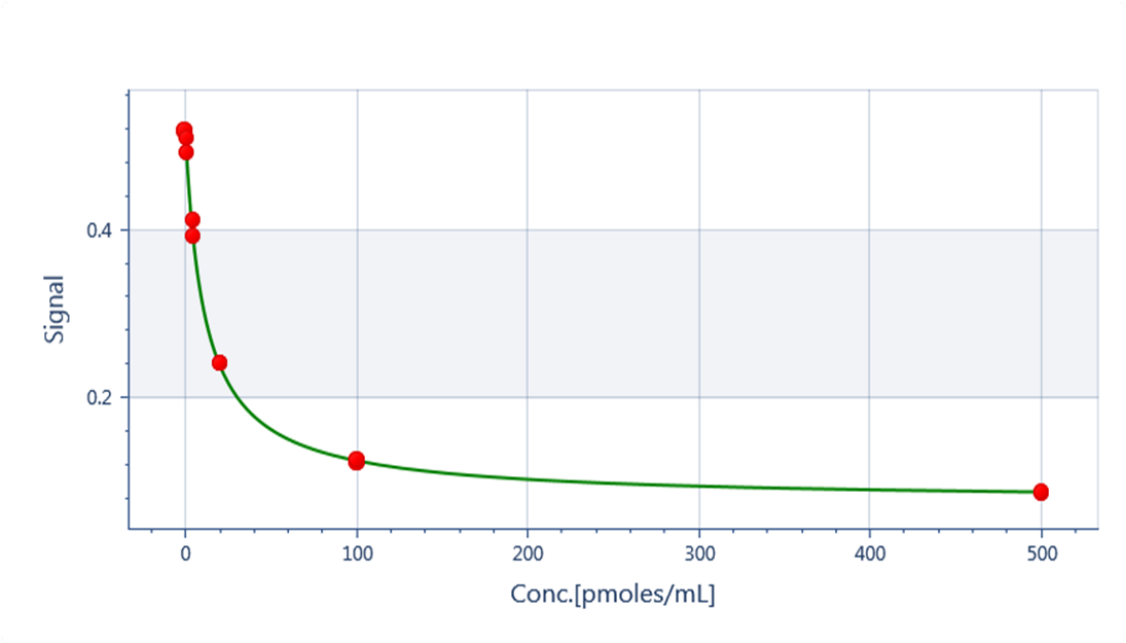


Figure 3.5A: Standard curve CGMP

Appendix 3. 6: A standard curve for the calibration and examples of peak in analysing plasma and urinary nitrate using chemiluminescence

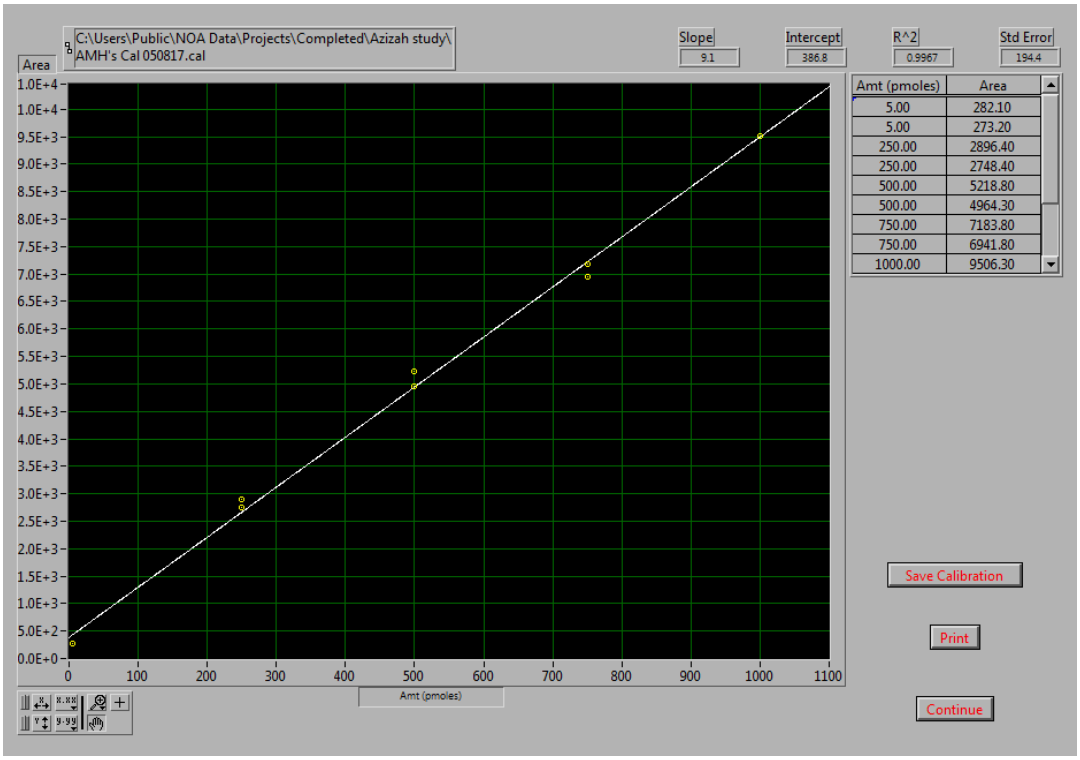


Figure 3.6 A: Standard curve plotted for the calibration in nitrate analysis using chemiluminescence

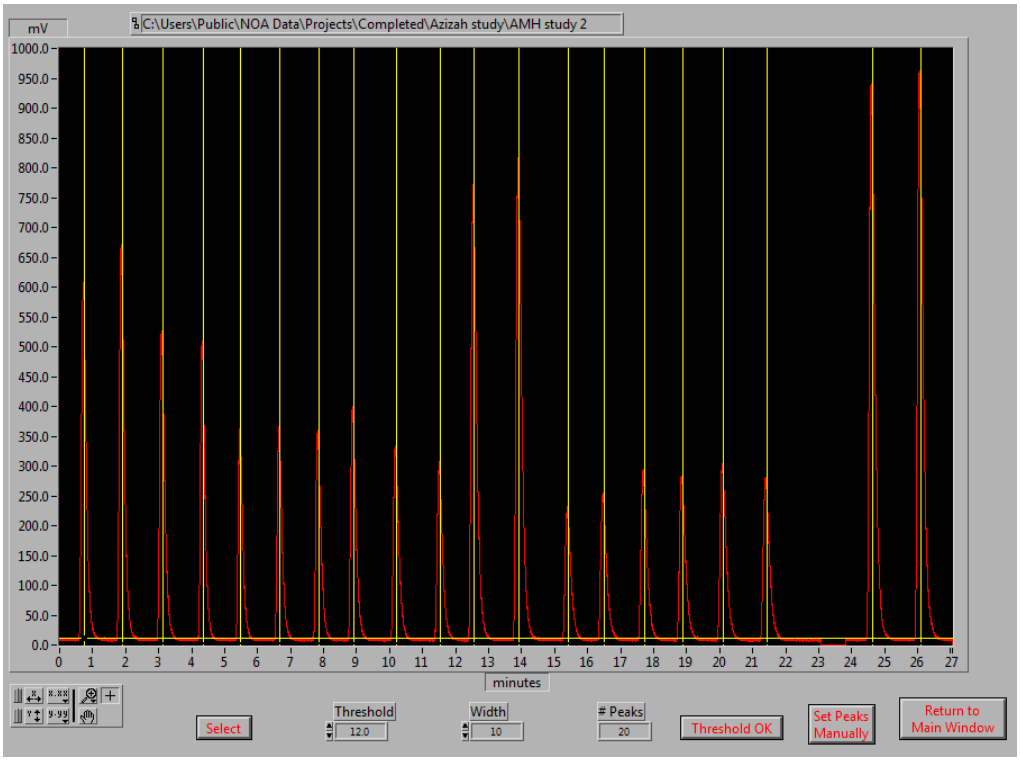


Figure 3.6 B: Example of plasma nitrate analysis using chemiluminescence

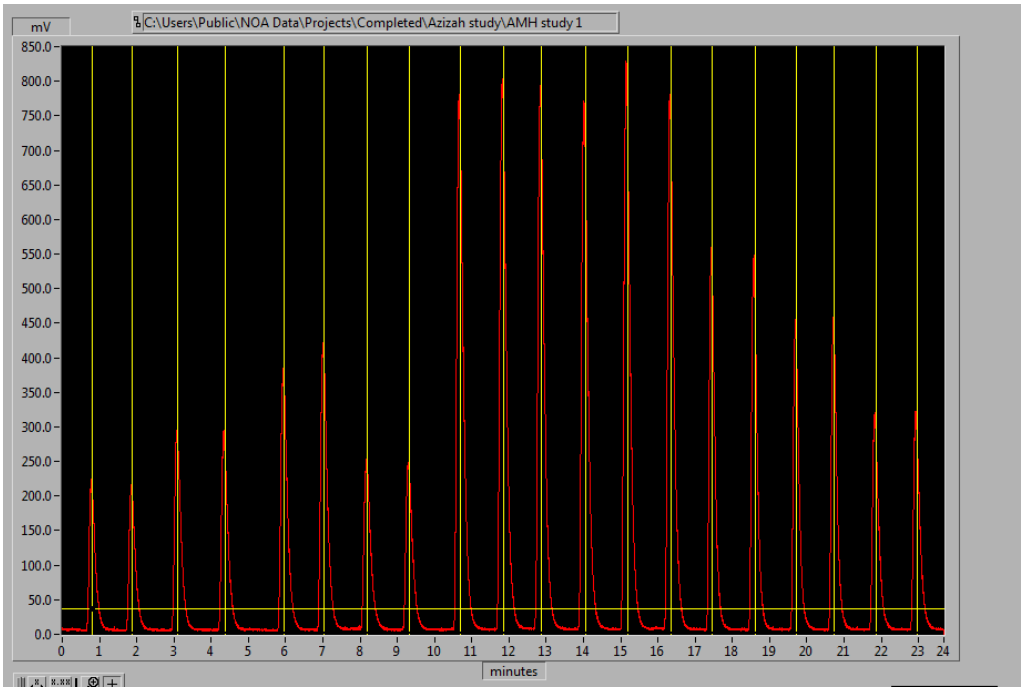


Figure 3.6 C: Example of urinary nitrate analysis using chemiluminescence

Appendix 3. 7: Performing NO_3^- analyses using chemiluminescence

Preparation of Nitrate Standard solutions

100mM NO_3 was prepared as a standard/stock solution for conducting a serial dilution to obtain the standard curve.

- 85mg of $NaNO_3$ was added in 10ml MiniQ H_2O to make 100mM NO_3 (standard solution).
- Seven tubes (marked with the following: 10mM, 1mM, 100µM, 10µM, 1µM, 100nM, 10nM) were arranged in a rack and 900µl MiniQ H_20 was added to each tube.
- Three tubes (marked with 50µM, 5µM, 50nM) were arranged in a rack and 300µl MiniQ H_20 was added to each tube.
- 100µl of stock solution was added to 10nM tube and was mixed and vortexed thoroughly. A new pipette tip was used to transfer 100µl of the 10mM standard to 1mM tube. These processes were repeated to prepare 10nM.
- 300µl of the 100uM standard was transferred to the 50µM tube, mixed and vortexed thoroughly. 300µl of the 10µM standard was transferred to the 5µM, mixed and vortexed thoroughly. 300µl of the 100µM standard was transferred to the 50nM, mixed and vortexed thoroughly.
- The most concentrated standards (10mM and 1mM) were discarded.
- To produce the standard curve, 20µL standards were injected (in duplicate) into the purge vessels (the most diluted standard was injected first), and the peak area of each standard were measured to produce NO_3 standard curves.

Preparation of Nitrate Reducing Agent

- 160mg of VCL_3 was weighted and resolved in 10ml MiniQ H_2O, then 10ml HCL was added to mixed and solved the pellet. The solution should be blue. This procedure was done in the hood. The solution was filtered using filter paper (Whatman #1)
- Antifoaming agent was diluted into 1:30. 100µL antifoaming agent was added into 3ml MiniQ H_2O.
- 1ml NaOH was added to 19ml of MiniQ H_2O to get 50% NaOH, then 15ml of this solution was added to the bubble base. This solution was used to prevent HCL vapors from entering the NOA.

General Operation Procedures

- Gas supplies (N_2 and O_2) to the NOA were switched on.
- NOA machine and PC were switched on. The machine was allowed to cool down to -12ºC.
- Once the temperature reached -12ºC, the supply pressure was 6psig and the cell pressure is >300 torr, the ENTER was pressed to returned to the main menu.
- The gas bubbler was opened to add 15ml of 1M NaOH to the bubble base.
- The bubbler top was replaced and sealed the bubbler by pressing the bottom onto the top and twisting to achieve a tight seal. The bubbler piece was secured with the green plastic clamp and mounted on the ring stand using the clamp.
- The IFD filter line was connected to the outlet of the bubbler.
- The bubbler line tubing was connected from the outlet of the purge vessel to the inlet of the bubbler.
- The gas inlet stopcock and the outlet stopcock on the purge vessel were closed and the needle valve was screwed on the purge vessel.
- The drain stopcock on the purge vessel was closed. The screw cap on the top of the purge vessel was opened and 3ml of the filtered VCL_3/HCL reagent was added.
- 200µl of the diluted antifoaming agent was added to the purge vessel and the screw cap was leaved off while adjusting the gas flow into the purge vessel.
- The gas inlet stopcock on the purge vessel was opened and the needle valve was opened slowly to start the flow of gas into the purge vessel. A slow, gentle bubbling of gas was obtained through the reagent.
- The screw cap was replaced, the outlet stopcock on the purge vessel was opened and the outlet stopcock on the gas bubbler was opened slowly while keeping an eye on the level of NaOH in the bubbler.
- To adjust the Purge Gas Flow rate, the IFD filter line was connected to the NOA's frit restrictor. The outlet stopcock on the purge vessel and on the gas bubbler was opened. The gas flow into the purge vessel was adjusted using the needle valve so that the cell pressure with the purge vessel were connected the same as recorded when the frit restrictor was open to the air (4 -7 torr).
- All stopcocks, power for the water bath and chiller were turned off after the experiments finished. Finally, the gas supplies were turned off.

Appendix 3. 8: Performing NO_2^- analyses using chemiluminescence

Preparation of Nitrite Standard solutions

100mM NO_2 was prepared as a standard/stock solution for conducting a serial dilution to obtain the standard curve.

- 69 mg of $NaNO_2$ was added in a 10mL MiniQ H_2O to make 100mM NO_3 (standard solution).
- Seven tubes (marked with the following: 10mM, 1mM, 100µM, 10µM, 1µM, 100nM, 10nM).
- Three tubes (marked with 50µM, 5µM, 50nM) were arranged in a rack and 300µl MiniQ H_20 was added to each tube.
- 100µl of stock solution was added to 10nM tube and was mixed and vortexed thoroughly. A new pipette tip was used to transfer 100µl of the 10mM standard to 1mM tube. These processes were repeated to prepare 10nM.
- 300µl of the 100uM standard was transferred to the 50µM tube, mixed and vortexed thoroughly. 300µl of the 10µM standard was transferred to the 5µM, mixed and vortexed thoroughly. 300µl of the 100nM standard was transferred to the 50nM, mixed and vortexed thoroughly.
- The most concentrated and diluted standards (10mM, 1mM and10nM) were discarded.

Preparation of Nitrite Reducing Agent

- 50mg of Nal was weighted and resolved in 2 ml MiniQ H_2O.
- 2ml of acetic acid, 500µl of Nal and 100µl diluted antifoaming were added to the purge vessel.

General Operation Procedures

- Gas supplies (N_2 and O_2) to the NOA were switched on.
- NOA machine and PC were switched on. The machine was allowed to cool down to -12ºC.
- Once the temperature reached -12ºC, the supply pressure was 6psig and the cell pressure is >300 torr, the ENTER was pressed to returned to the main menu.
- The drain stopcock, gas inlet stopcock and outlet stopcock on the purge vessel were closed. The septum and cap from the top of the purge vessel was removed. The needle valve on the purge vessel was screwed fully to stop the gas flow.

- 2ml of concentrated acetic acid was added to the purge vessel. With the needle valve fully closed, the gas inlet stopcock on the purge vessel was opened. The needle valve was opened slowly to allow gas to flow into the acetic acid. A slow, gentle bubbling of gas was obtained through the acid and any dissolved oxygen was removed. 500µl Nal was added to the purge vessel. The screw cap and septum on the purge vessel were installed.
- IFD filter line was connected to the frit restrictor and the nut was tighten finger tight. The outlet stopcock was opened and the gas flow into the purge vessel was adjusted using the needle valve to keep the cell pressure around 4 to 7 torr.
- All stopcocks, power for the water bath and chiller were turned off after the experiments finished. Finally, the gas supplies were turned off.

Appendix 3. 9: Performing Nitrate Colorimetric Assay

Performing the Griess Reaction

A commercial kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical, Ann Arbor, MI, US) was used to measure NO_3^-concentrations. The following components were required in order to perform the analysis: 1) Nitrate Assay Buffer; 2) Nitrate Reductase Enzyme Preparation; 3) Nitrate Reductase Cofactors Preparation; 4) Nitrate Standard and 5) Griess Reagent Reagents.

Sample Preparation

Plasma samples were filtered through 0.5mL centrifugal filters (Amicon ®Ultra, Merck Millipore, Cork, IRL). The filters were pre-rinsed with UltraPure water prior to ultrafiltration of the plasma (centrifudged at 14,000 rpm for 7 minutes). Plasma was diluted 1:2 in assay buffer.

Plate set up

At least two wells must be designated for absorbance blanks (containing 200 µL of Assay Buffer or water). The absorbance of these wells was then subtracted from the absorbance measured in all other wells. Standard curves for NO_3^- were obtained for the calculation of the concentrations.

Measurement of Nitrate: Assay procedure

In this procedure, all samples and standards were assayed in duplicate. A plate map was used to record the location of standards and samples.

The following steps were followed to perform the assay:

1) 200 µL of assay buffer were added to the blank wells.
2) 80 µL of diluted samples was added to each of the sample wells.
3) 10 µL of the enzyme cofactor and reductase mixture were added to each of the wells.
4) The plate was covered and incubated at room temperature for one hour.
5) After the required incubation time, 50 µL Griess reagents 1 and 2 were added to each well. The colour in the plate was allowed to developed for 10 minutes before the absorbance was read at 540 nm using plate reader (Multiskan Go Plate Reader, Thermo Scientific).

Determination of sample concentrations

The calculation of nitrate concentrations involved the subtraction of the absorbance value of the blank wells from the absorbance values from all the other wells. A plot of absorbance at 540-550 nm as a function of NO_3^- concentration was made. The NO_3^- standard curve was used for the determination of total nitrate + nitrite concentration. The following algorithm were used for determination of NO_3^-.

$$[\text{Nitrate + Nitrite}]\ (\mu M) = \left(\frac{A_{540} - \text{y-intercept}}{\text{slope}}\right)\left(\frac{200\ \mu l}{\text{volume of sample used } (\mu l)}\right) \text{ x dilution}$$

Sensitivity

When using the maximum amount of sample for the nitrate assay (80 µl), the detection limit was 2.5 µM. The detection limit for the plasma was higher since only 40 µl of sample can be used.

Appendix 3. 10: Procedure of nitrate-derivatisation for GCMS analyses

Chemicals and reagents

Labelled sodium nitrate ($^{15}NaNO_3$, 98$^+$ atom %, Cambridge isotope laboratories, Inc., Andover, USA) was obtained from Sigma Aldrich Co, USA; unlabelled sodium nitrate ($^{14}NaNO_3$) was obtained from Fisher Scientific, Loughborough, UK; mesitylene, TFAA, sodium sulphate, sodium bicarbonate was all obtained from Sigma-Aldrich, UK. Deionised water was used for the aqueous solutions and during the dilution preparation.

Safety

The work has been done in biological safety cabinet due to some of the chemicals that are harmful as follows:

1) Sodium Nitrate: Harmful if swallowed and can cause irritation to the skin, eye and respiratory tract.
2) Mesitylene: Flammable liquid and harmful if swallowed or inhaled as it can be evaporated. Can cause irritation to skin and respiratory tract, toxic to aquatic life with long lasting effects.
3) TFAA: Can cause severe skin burns and eye damage. Harmful if inhaled and it can react aggressively with water.

Standards Preparation

A series of experiments using different concentrations and enrichments of $NaNO_3^-$ was performed to investigate the accuracy of the methods and to gain proficiency of conducting the experiment before the real samples were analysed.

The first experiment was performed to explore the linearity of measurements of $Na^{14}NO_3^-$ in aqueous solution across different range of concentrations. The concentrations range from 5 mmol/l to 0.078 mmol/l. The volume of the samples was 500µl.

The following formula was used to prepare the sample of $Na^{14}NO_3^-$ (5mmol/L) and $Na^{15}NO_3^-$ (5mmol/L) solution.

[^{14}N] Sodium Nitrate ($NaNO_3$): molar mass is 84.99 g/mol

[^{15}N] Sodium Nitrate ($NaNO_3$): molar mass is 85.99 g/mol

$$\text{Weight (mg)} = \frac{(5mmol/L)*molar\ mass*Volume(100ml)}{1000}$$

- 42.5 mg of sodium nitrate (for ^{14}N**) /** 43 mg of sodium nitrate **((**for ^{15}N) were weighted on the sensitive balance and transferred to a small beaker before being dissolved in a small amount of deionized water.
- The solution was transferred into a clean 100mL volumetric flask and make up to 100 mL with deionized water and mix thoroughly.

The following steps were followed to perform the experiment.

1. The samples were thawed at the room temperature.
2. The samples were transferred to Agilent 1.5 mL GCMS vial (the volume of the samples may be varies).
3. The samples were dried down under nitrogen at room temperature. This procedure took approximately 60 to 120 minutes- depending on the volume of the samples in the vials.
4. After this procedure, 200µL TFAA and 1mL mesitylene were added to the dried samples.
5. The samples were incubated at 70ºC for 1 hour in heating block, and then allowed to cool down at room temperature.
6. Four clean disposable culture tubes were prepared (per sample) and labelled appropriately on the rack. 500mg of sodium sulphate (Na_2SO_4) was added in the fourth (last) culture tube for each sample.
7. The samples in the vials (containing TFAA and mesitylene) were then transferred to the first culture tube.
8. One ml de-ionised water was added (slowly) to the samples and vortex for 10 seconds. This procedure resulted the separation of 2 layers of solvent in the tube.
9. The top solvent layer was transferred to the second culture tube.
10. One ml of 1% aqueous sodium bicarbonate (MW 84; 84mg $NaHCO_3$ in 100mL of deionised water) was added to the second culture tube, vortexed for 10 seconds and the top layer was transferred to the third culture tube.
11. One ml de-ionised water was added to the third culture tube and vortex for 10 seconds and the top layer were transferred to the fourth culture tube (containing the sodium sulfate).
12. Pre-plugged Pasteur pipettes were prepared by flushing through with 500µL mesitylene.
13. The samples from the final culture tubes were removed and filtered through the prepared Pasteur pipette into a clean labelled 1.5 mL GCMS vials.

14. The solutions are now ready to be analysed by GCMS.

The coefficient of determination showed good linearity of the method ((R^2= 0.9911) which was maintained in the lowest concentrations (0.0078) (**Figure 3.13**).

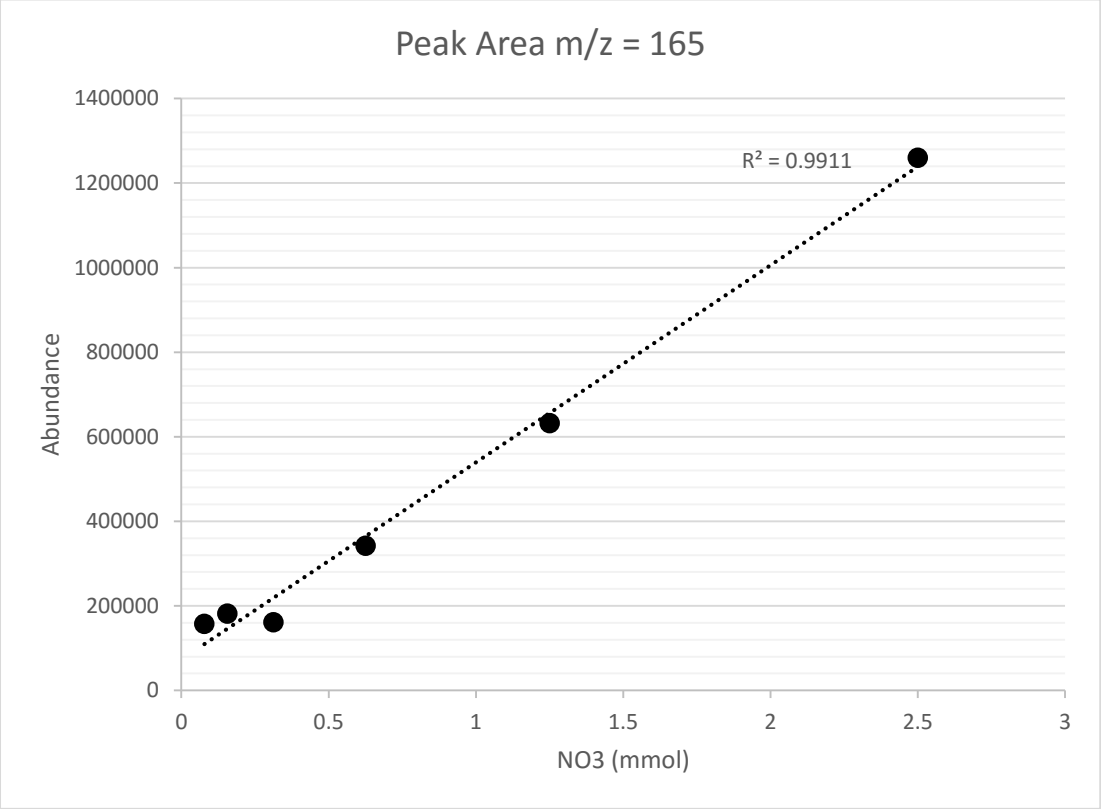


Figure 3.13: Regression analysis of peak areas of the ion 165 versus NO_3 concentration in an unlabelled serial dilution of $Na^{14}NO_3$ standards.

The second experiment was performed to investigate the performance of the nitro-mesitylene method in aqueous solution in different range of concentrations (5 mmol/l to 0.078 mmol/l). In a clean Agilent vials, 50 µl of each unlabelled $Na^{14}NO_3^-$ was spiked with 50 µl of $^{15}NO_3^-$ to produce a range of enrichments. The total volume of the samples was 100 µl. The starting unlabelled and labelled baseline solutions of $NaNO_3^-$ are described in **Table 3.2**.

Table 3.2: Concentration (mmol/l) and total nitrate (NO_3^-) content (mg/ml) of the labelled and unlabelled NO_3^- aqueous solutions.

	Sample	Total Nitrate Content (mg/ml)	Total Nitrate Content (mg/l)	Concentration (mmol/l)	Molecular mass (gr)
Unlabelled, $Na^{14}NO_3^-$	A	0.425	42500	5	85
Labelled, $Na^{15}NO_3^-$	B	0.025	2494	0.29	86

The coefficient of determination (R^2) was 0. 9966 as shown in **Figure 3.14.**

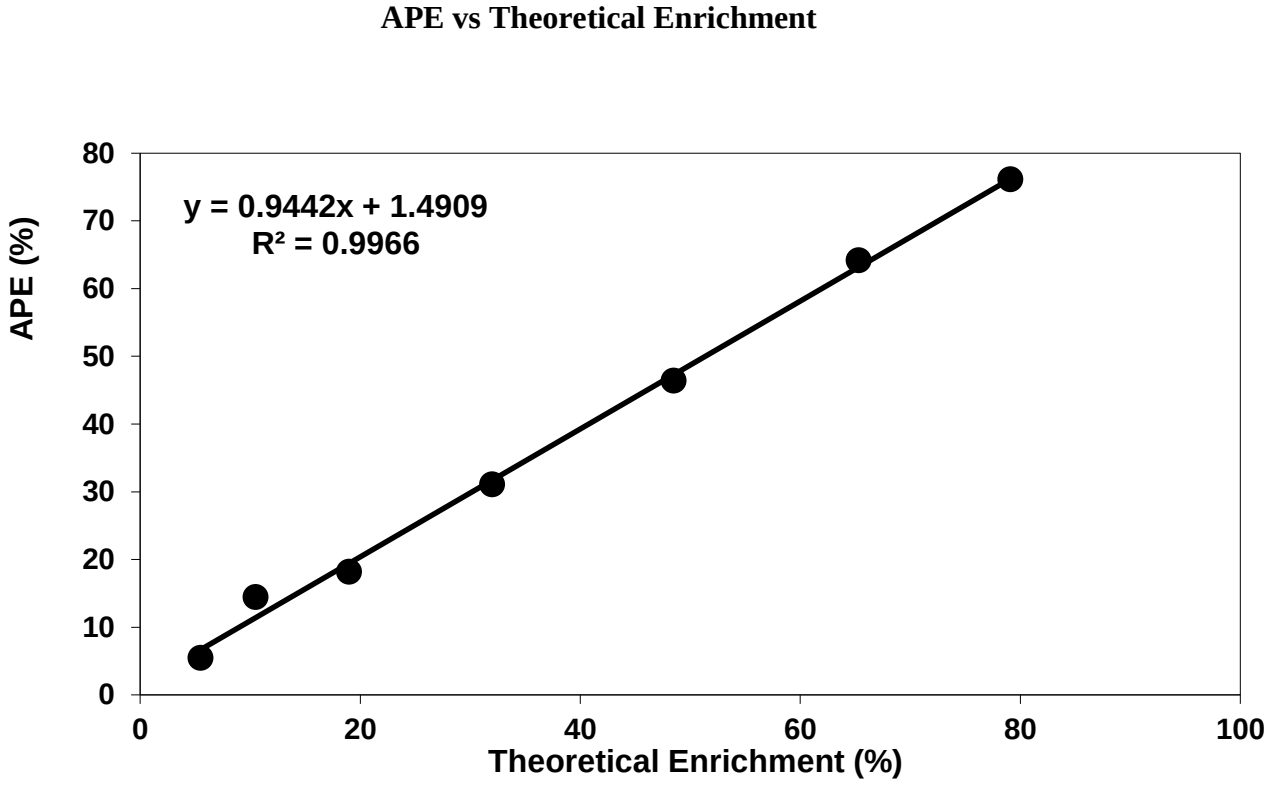


Figure 3.14: Changes in isotopic enrichment in a serial dilution of unlabelled $Na^{14}NO_3^-$ standards spiked with 0.29 mmol $Na^{15}NO_3$.

The third experiment investigated the agreement between theoretical and measured enrichments in $^{15}NO_3^-$ using a range of enrichments in spiked standard aqueous solutions having the same concentration (5mmol/l). In this experiment, both $^{15}NO_3^-$ and $^{14}NO_3^-$ are 5mmol/l. The samples had a total volume of 100 µl for each vials **(Table 3.3).**

Table 3.3: Isotopic enrichment in a 5 mmol aqueous solution of $Na^{14}NO_3$ spiked with different amounts of a 5 mmol solution of $Na^{15}NO_3$

	Isotopic Enrichment (%)	$Na^{14}NO_3$ (5mmol) (µl)	$Na^{15}NO_3$ (5mmol) (µl)	$Na^{14}NO_3$ (5mmol) (µg/ml)	$Na^{15}NO_3$ (5mmol) (µg/ml)	Final Concentration (mmol/l)
1	100	0	100	0	0.43	5
2	50	50	50	0.42	0.43	5
3	10	90	10	0.42	0.43	5
Serial Dilution	5	100	100 (IE 10%)	0.42	0.43	5
	2.5	100	100 (IE 5%)	0.42	0.43	5
	1.25	100	100 (IE 2.5%)	0.42	0.43	5
	0.625	100	100 (IE 1.25%)	0.42	0.43	5
	0.313	100	100 (IE 0.625%)	0.42	0.43	5
9	0	100	0	0.42	0	5

The coefficient of determination (R^2) was 1 as shown in **Figure 3.15**.

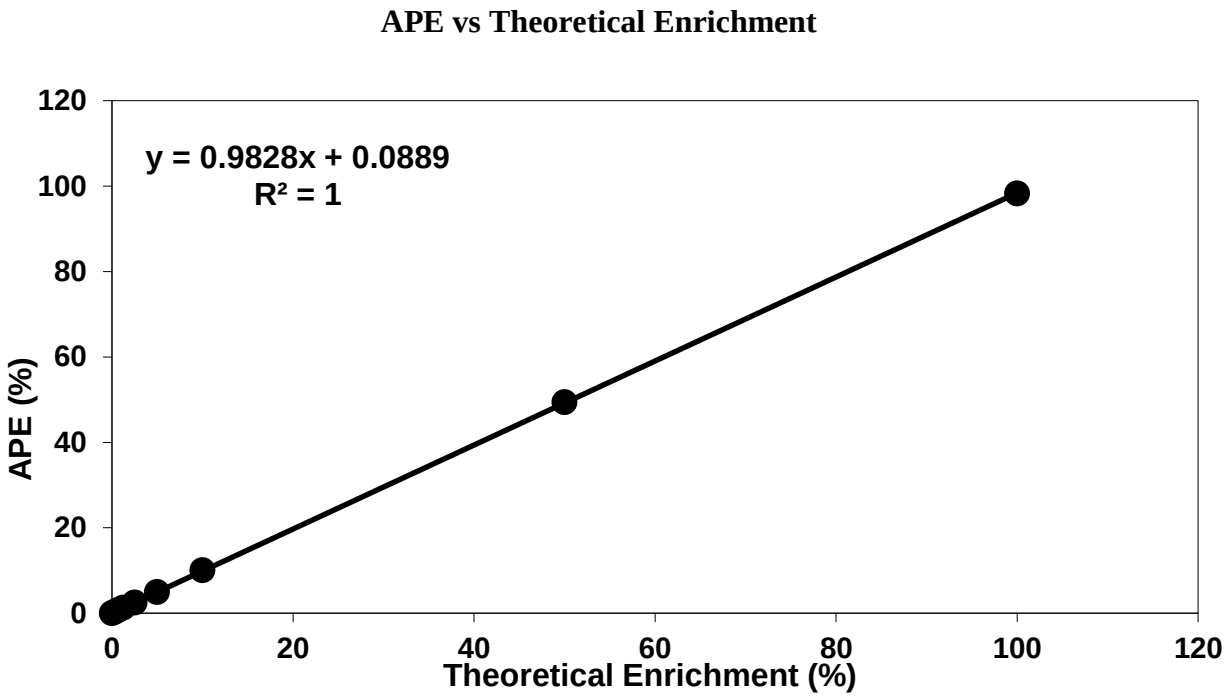


Figure 3.15: Regression analysis of peak areas of the ion 165 versus NO_3 concentration in an unlabelled serial dilution of $Na^{14}NO_3$ standards.

Appendix 3. 11: GCMS data analyses

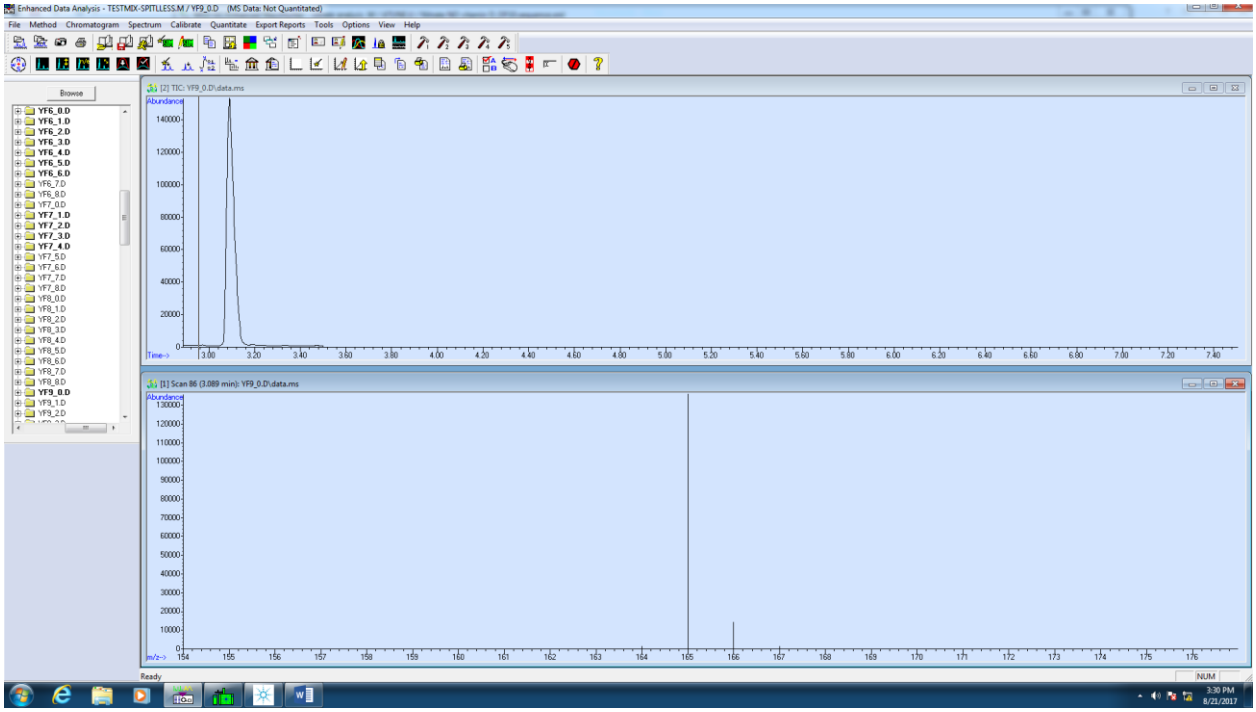


Figure 3.11A: Example of GCMS chromatogram and ion spectrum before the administration of the labelled nitrate dose

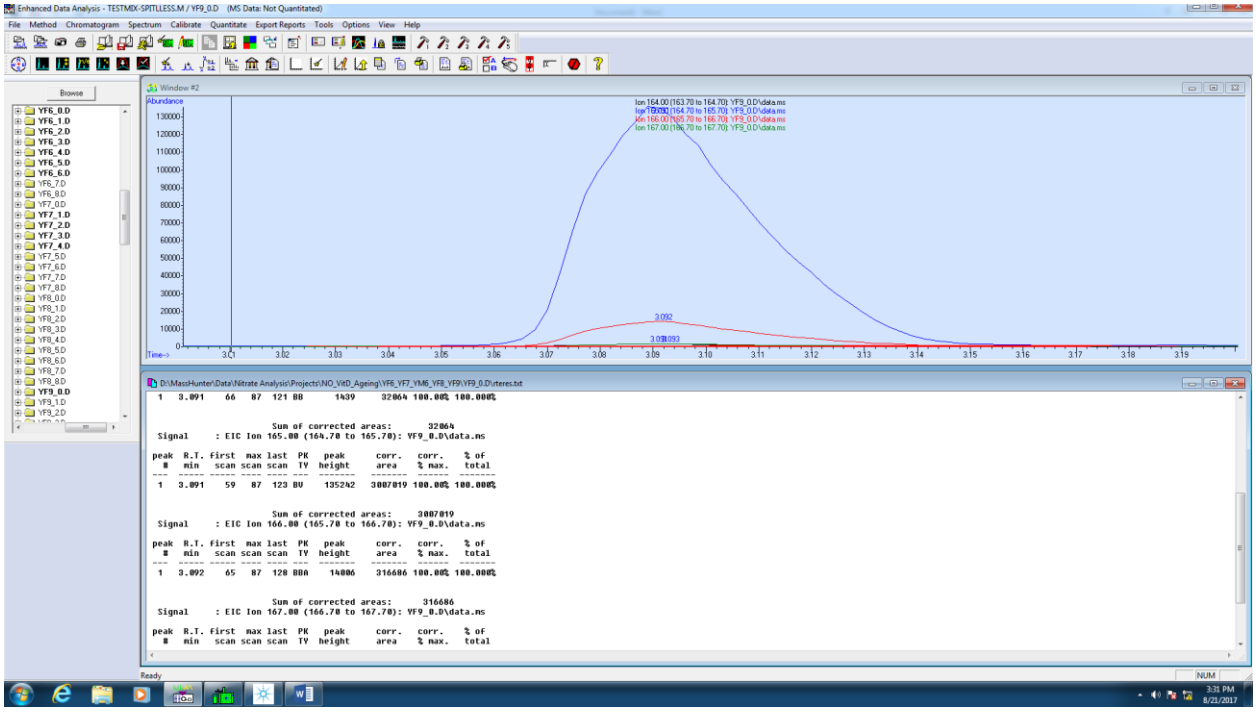


Figure 3.11B: Example of integrated chromatogram for 165 and 166 ions

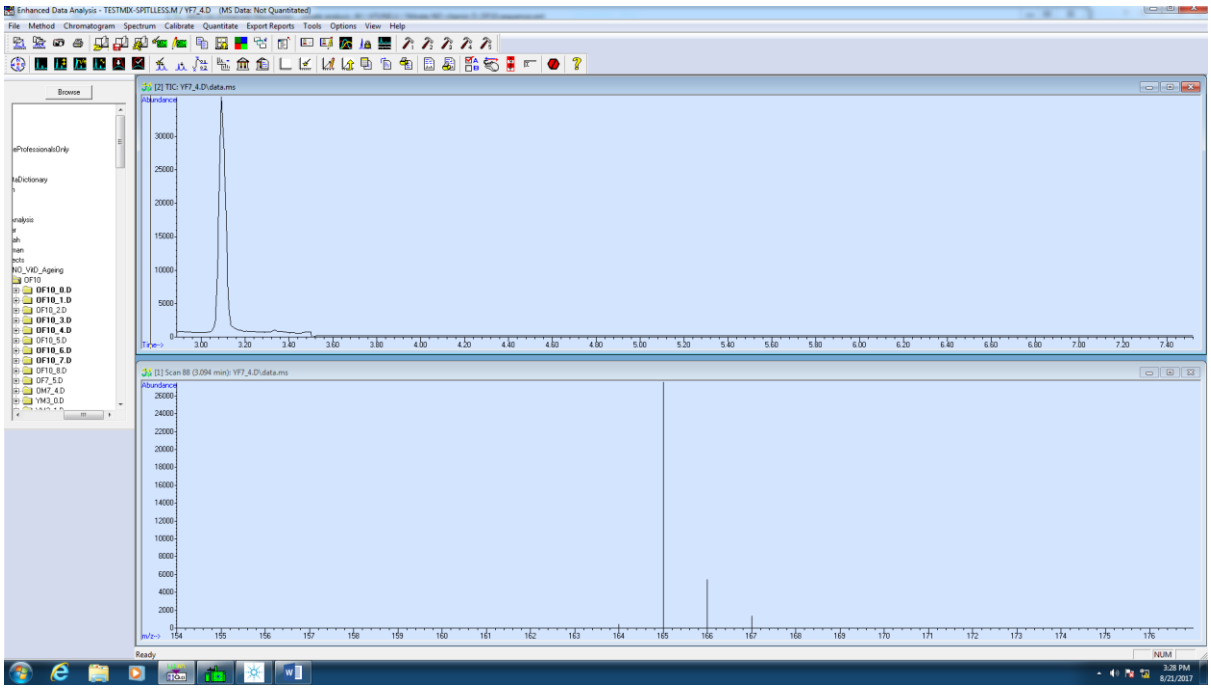


Figure 3.11C: Example of GCMS chromatogram and ion spectrum after the administration of the labelled nitrate dose

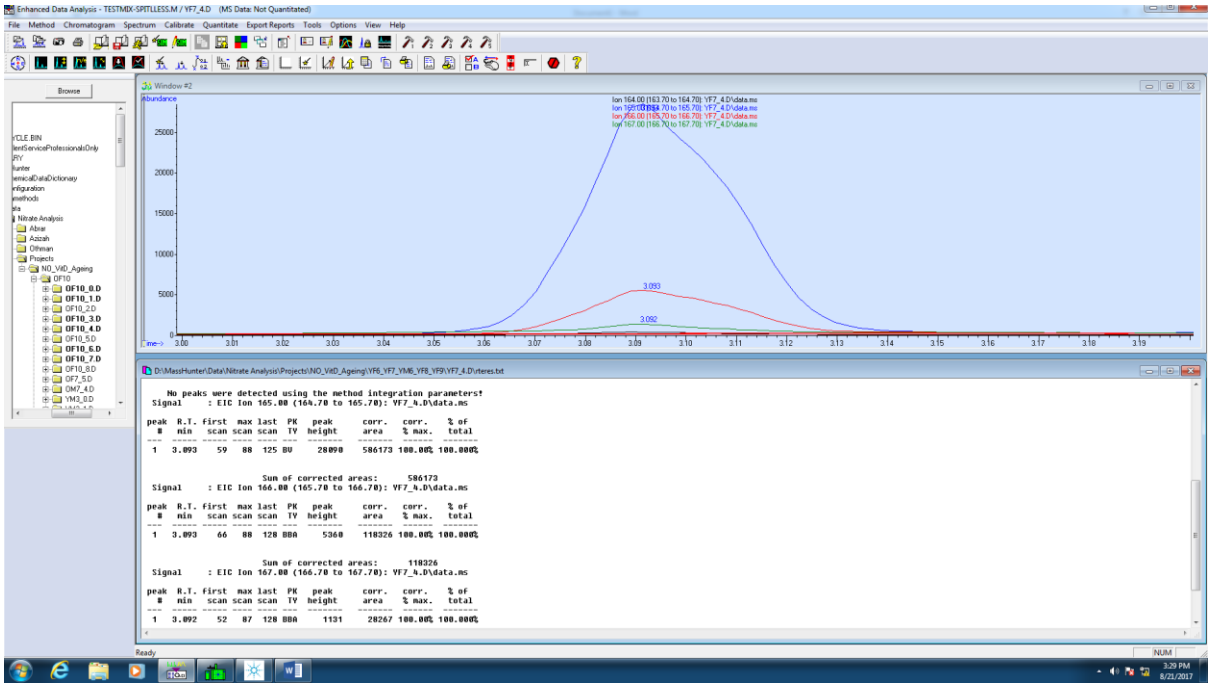
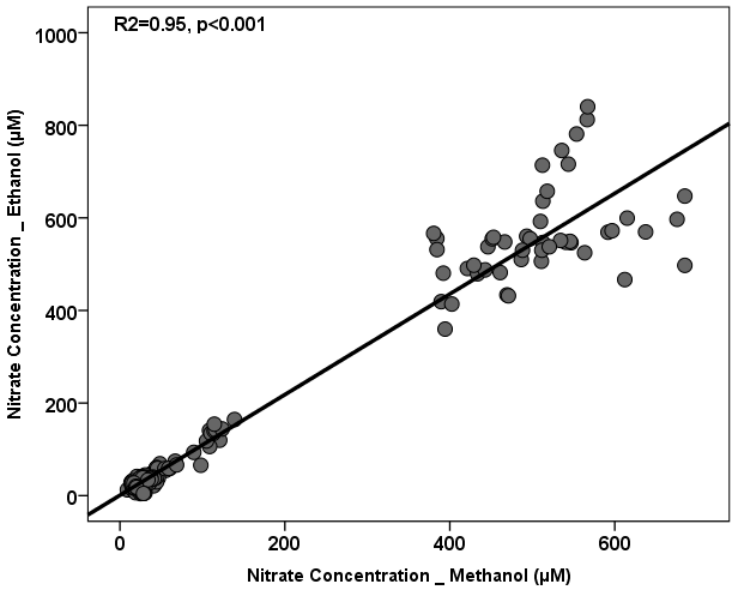
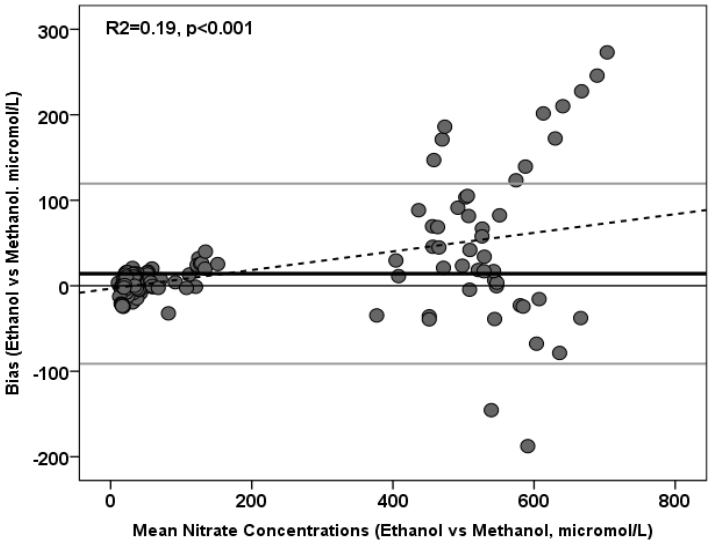


Figure 3.11D: Example of integrated chromatogram for 165 and 166 ions

Appendix 3. 12: Comparison of CL for deproteination method using ETH and MTH (after removal of the outliers).



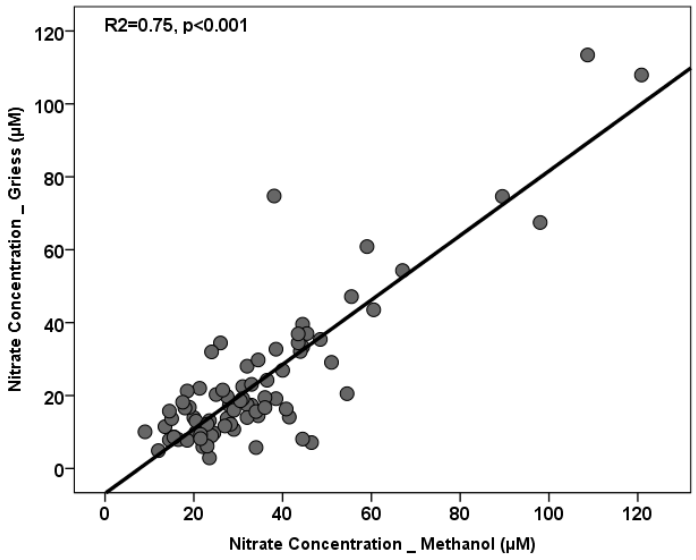
(B) Regression analysis of plasma nitrate concentration (µM) estimated by CL after deproteination using the ETH and MTH methods (after the removal of the outliers)



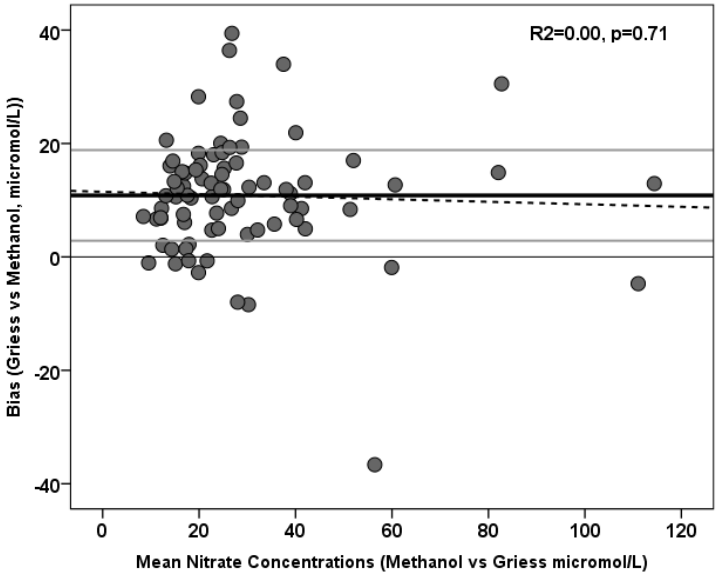
(B) Bland-Altman analysis of estimates of plasma nitrate concentration after deproteination using the ETH and MTH methods (after the removal of the outliers)

Figure 3.9A: Comparison of CL for deproteination method using ETH and MTH (after removal of the outliers). (A) Linear regression analysis between plasma nitrate concentration observed by the ETH and MTH. (B) Plotting according the method of B & A agreement between the difference in the nitrate concentrations measured by the ETH and MTH. Black horizontal lines (bold) show the mean difference between ETH and MTH and the fine, grey line, are the limits of agreement (± 2SD).

Appendix 3. 13: Comparison of CL and Griess methods for the measurements of plasma concentrations



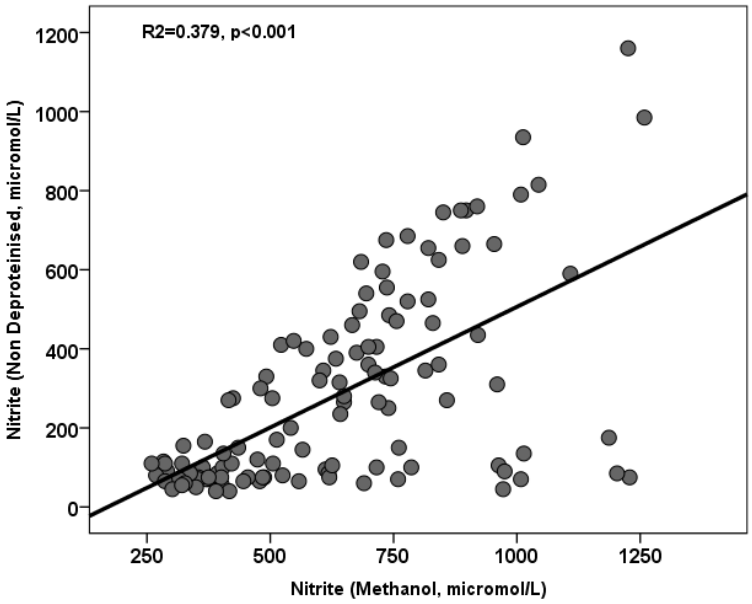
(A) Regression analysis of plasma nitrate concentration (µM) estimated by Griess and MTH



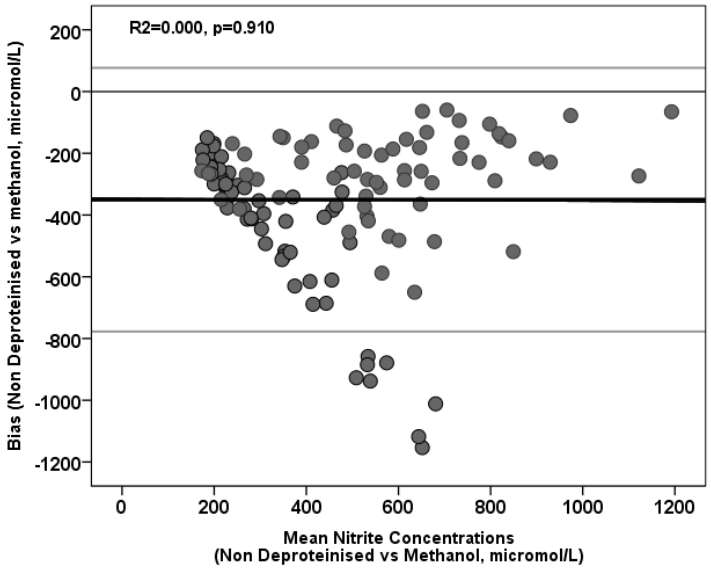
(B) Bland-Altman analysis of estimates of mean plasma nitrate concentration using Griess and MTH methods

Figure 3.10A: Comparison of CL and Griess methods for the measurements of plasma concentrations in 80 subjects. (A), Linear regression analysis to evaluate the association between plasma nitrate concentration measured by the Griess and CL methods. (B), Agreement and differential bias between the two methods was evaluated by the Bland-Altman method. Black horizontal lines (bold) show the mean difference between CL and Griess methods and the fine, grey line are the limits of agreement (± 2SD). A regression line was fitted to the points (dashed black line) to evaluate differential bias. Plasma samples measured by CL were processed using the MTH method.

Appendix 3. 14: Comparison of plasma nitrite measured by CL in untreated samples and using the MTH deproteination method



(A) Regression analysis of plasma nitrite concentration (µM) estimated by CL (untreated vs MTH)



(B) Bland-Altman analysis of estimates of mean plasma nitrite concentration using CL (untreated vs MTH)

Figure 3.11A: Comparison of plasma nitrite measured by CL in untreated samples and using the MTH deproteination method. (A), Linear regression analysis to evaluate the association between the two processing methods. (B), B & A to assess agreement between the two methods and evaluate presence of differential bias. Black horizontal line shows the mean difference (bold) and the ± 2S.D. range (fine, grey line). A regression line was fitted to the points (dashed black line) to evaluate differential bias.

Appendix 3. 15: Procedure of analysing of 25(OH)D concentration using conventional method

It involved three phases of incubation. In the first incubation, 15uL of sample was incubated with pretreatment reagent 1 and 2, resulted in the releasing bound 25OH vitamin D from the vitamin D binding protein. In the second incubation, the pretreated sample was incubated with ruthenium labelled vitamin D binding protein which leads to the formation of a complex between the vitamin D and the ruthenylated vitamin D binding protein. In the third incubation, the addition of streptavidin-coated microparticles and 25(OH)D labelled with biotin and the unbound ruthenium labelled vitamin D binding proteins become occupied. A complex consisting of the ruthenylated vitamin D binding protein and the biotinylated 25-OH vitamin D was formed and became bounded to the solid phase via interaction of biotin and streptavidin.

The reaction mixture was then aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. The unbound substances were then removed. The application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

After separation, the samples stability was depending on the temperature where it was kept. The analytical range of CLIA method were between 7.50 to 175 nmol/L and samples with the concentration outside these limits are reported as <10nmol/L or >174nmol/L. Coefficient of Variability (CV) for inter-assay analyses is 18.4% at a 25(OH)D level of 39.5 nmol/L and 11.7% at 121.25 nmol/L.

Appendix 3. 16: Procedures of analysing 25(OH)D using dry blood spot (DBS) method

Technical Service Information Sheet

Determination of 25-Hydroxyvitamin D_3 & D_2 in Dried Blood Spots by LC-MS/MS

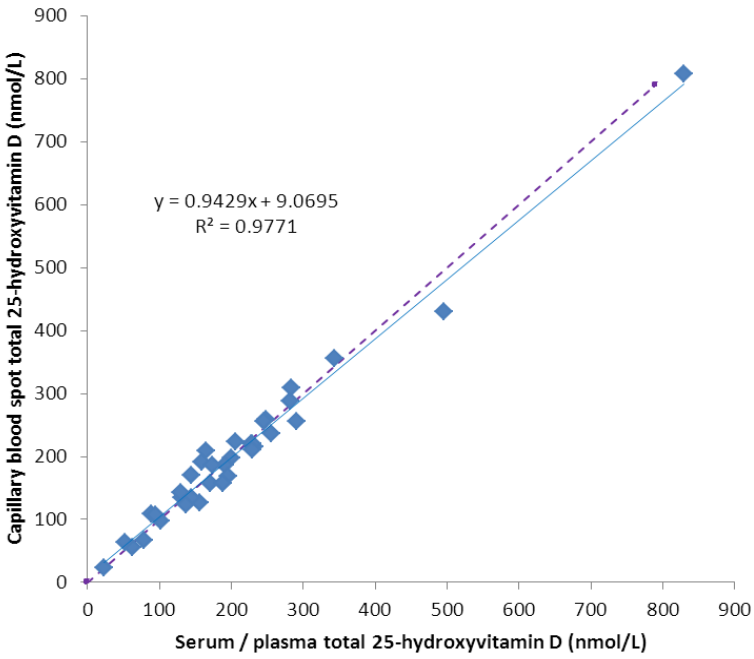
Samples

Suitable collection kits and blood spot devices are supplied as part of the testing process. Samples show good stability and may be sent via the postal system using the envelope provided in the kit.

The blood spot method involved taking a 3mm punch from a dried blood spot. The punches were extracted and the 25-hydroxyvitamin D were derivatived with 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD), which increased the sensitivity during MS-detection. After some further sample preparation, the samples were loaded onto liquid chromatography–mass spectrometry (LC/MS/MS) analyser (Waters i-Class UPLC and Waters Xevo TQ-S triple quad mass spectrometer). Multiple reaction monitoring (MRM) mass spectrometry was used to analyse the samples for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 and these total were added to report total 25-hydroxyvitamin D.

The precision and accuracy of this method were reported by City Assay as below:

Parameter	Blood spot vitamin D
Linearity	$r^2 > 0.98$
Limit of quantitation (CV <20%)	< 7.1 nmol/L
Intra assay variation	<10 %
Inter assay variation	<11 %



Method

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method is applied to dried blood spot samples, utilising blood spot calibrators. The method is standardised against our conventional 25-hydroxyvitamin D_3 and D_2 LC-MS/MS service for plasma/serum. The laboratory participates in the DEQAS external quality assurance scheme. Blood spot results show good comparability to serum/plasma results.

***Figure 1**: Comparison of total 25-hydroxyvitamin D results for capillary blood spots with conventional LC-MS/MS method for paired serum or plasma samples (n = 36), data for samples from October 2012 until May 2015*

Reference ranges

Total 25-Hydroxyvitamin D status	Concentration (nmol/L)
Severe deficiency	<15
Deficiency	15 – 30
Insufficiency	30.1 – 50
Adequate	50.1-220
High to toxic	220.1-500
Toxic	>500

The individual values for 25-hydroxyvitamin D_3 and D_2 are reported as well as the total in nmol/L. Target turnaround time is 2-3 days. Results are reported with a clear interpretation and sent via first class post or a PDF report is emailed to the specified email address.

Appendix 4. 1: Summary findings of observational studies on vitamin D and cardiovascular disease

No	Author	Study design	Country	Main Outcome
1.	Michal L. Melamed (Melamed et al., 2008)	Cross sectional	US	25(OH)D levels were inversely associated with hypertension, diabetes mellitus, hypertriglyceridemia, and obesity
2.	Harald Dobnig (Dobnig et al., 2008)	Prospective cohort	Germany	Low 25(OH)D levels correlated inversely with markers of inflammation (C-reactive protein and interleukin-6), oxidative burden (serum phospholipid and glutathione), and cell adhesion (vascular cell adhesion molecule-1 and intercellular adhesion molecule-1)
3.	Jeffrey L. Anderson (Anderson et al., 2010)	Prospective study	US	Serum 25(OH)D levels <30 ng/ml were associated with highly significant increases in the prevalence of diabetes, hypertension, hyperlipidemia, and peripheral vascular disease. Serum 25(OH)D levels were also highly associated with coronary artery disease, myocardial infarction, heart failure and stroke
4.	Suzanne E Judd (Judd et al., 2008)	Cross sectional	US	25(OH)D levels were inversely associated with hypertension, diabetes mellitus, hypertriglyceridemia, and obesity
5.	Edward Giovannucci (Giovannucci et al., 2008)	Prospective Study	US	Men deficient in 25(OH)D (<15 ng/ml) were at increased risk for myocardial infarction compared with those considered to be vitamin D sufficient (>30 ng/ml)
6.	Archna Bajaj (Bajaj et al., 2014)	Follow up study	US	Significant association between circulating 25(OH) vitamin D and risk of CVD events
7.	Lu Wang (Wang et al., 2013)	Prospective study	US	Inverse relationship between vitamin D levels and development of hypertension
8.	Yakup Alsancak (Alsancak et al., 2015)	Prospective cohort	Turkey	Failed to demonstrate significant relationship between serum vitamin D levels and the severity and extent of coronary artery disease
9.	Thomas J. Wang (Wang et al., 2008)	Longitudinal study	US	Vitamin D deficiency is associated with incident cardiovascular disease.

No	Author	Study design	Country	Main Outcome
10.	Scragg, R (Scragg *et al.*, 2007)	Cross sectional	US	Vitamin D status was associated inversely with BP.
11.	Judd, S,E (Judd *et al.*, 2008)	Cross sectional	US	25(OH)D levels inversely associated with hypertension, diabetes mellitus, hypertriglyceridemia, and obesity
12.	Martins, D (Martins *et al.*, 2007)	Prospective cohort	US	Serum 25(OH)D levels are associated with important cardiovascular disease risk factors
13.	Hintzpeter, B (Hintzpeter *et al.*, 2008)	Prospective cohort	German	The results demonstrate that moderate and mild vitamin D deficiency affects a large proportion of the adult population in Germany
14.	Hypponen, E (Hypponen *et al.*, 2008)	Prospective cohort	UK	Serum 25(OH)D is inversely associated with metabolic syndrome, whereas the inverse association with IGF-1 was found only among those without hypovitaminosis D. These results suggest that metabolic syndrome prevalence is the lowest when both 25(OH)D and IGF-1 are high.
15.	Forouhi, N.G (Forouhi *et al.*, 2008)	Prospective study	UK	Inverse associations between baseline serum 25(OH)D and glycemia and insulin resistance
16.	Reis, J.P (Reis *et al.*, 2007)	Prospective cohort	US	An increased risk of metabolic syndrome with elevated PTH levels in older men
17.	Gannage-Yared, M.H (Gannage-Yared *et al.*, 2009)	Cross sectional	Lebanon	There are relationships between 25(OH)D and several metabolic risk factors and adiponectin
18.	Pasco, J.A (Pasco *et al.*, 2009)	Cross sectional	Australia	Women with high vitamin D were less likely to have elevated PTH, hypertension or bone deficits than women with poor levels.
19.	Rueda, S (Rueda *et al.*, 2008)	Cross sectional	Spain	The findings did not support an independent contribution of 25(OH)D or PTH in the pathogenesis of the metabolic syndrome in severely obese subjects
20.	Chan, R (Chan *et al.*, 2012)	Cross sectional	China	The findings support an association between serum PTH and blood pressure, but not for serum 25(OH)D in older Chinese men whose vitamin D status is optimal.
21.	Williams, D.M (Williams *et al.*, 2011)	Cross sectional	US	Higher calcium levels might be a more important predictor of increased cardiovascular risk in adolescents than lower 25(OH)D levels or PTH levels

No	Author	Study design	Country	Main Outcome
22.	Dorjgochoo, T (Dorjgochoo *et al.*, 2012)	Cross sectional	China	Circulating 25(OH)D levels were inversely related to levels of individual BP parameters and hypertension among middle-aged and elderly men but not in women
23.	Snijder, M.B (Snijder *et al.*, 2007)	Cohort study	Netherlands	Serum 25(OH)D was not associated with blood pressure,
24.	Jablonski, K.L. (Jablonski *et al.*, 2011)	Cross sectional	US	Inadequate serum 25(OH)D is associated with vascular endothelial dysfunction among healthy middle-aged/older adults and this is mediated in part by NFκB-related inflammation.
25.	Yiu, Y.F (Yiu *et al.*, 2011)	Cross sectional	China	Serum 25(OH)D status was significantly associated with brachial artery FMD
26.	Codoner-Franch, P. (Codoner-Franch *et al.*, 2012)	Cross sectional	Spain	Insufficient 25(OH)D levels were detected in severely obese children with increased markers of oxidative/nitrosative stress, inflammation, and endothelial activation.

Appendix 4. 2: Study sponsorship

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

AJ/NG/JH

Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne
NE1 4LP

Tel: 0191 233 6161
Fax: 0191 201 0155
www.newcastle-hospitals.nhs.uk

11 November 2014

Professor David Burn
Director, Institute of Neuroscience & Professor of Movement Disorder Neurology
Campus of Ageing and Vitality
Newcastle University
NE4 5PL

Dear Professor Burn

Trust R&D Project:	**7074**
Title of Project:	**Cross-sectional study to evaluate the association between Vitamin D status and endothelial function in obese, post-menopausal women**
Principal Investigator:	**Professor David Burn**
Number of patients:	**80**
Funder (proposed):	**MARA (Majilis Amanah Rakyat)**
Sponsor (proposed):	**The Newcastle upon Tyne Hospitals NHS Foundation Trust**
REC number:	**14/EM/1073**
IRAS Project Code:	**152807**
First participant to be recruited by:	**11 December 2014**

After completing the necessary risk and site assessment for the above research project, The Newcastle upon Tyne Hospitals NHS Foundation Trust grants NHS Permission for this research to take place at this Trust dependent upon:

(i) you, as Principal Investigator, agreeing to comply with the Department of Health's Research Governance Framework for Health and Social Care, and confirming your understanding of the responsibilities and duties of Principal Investigators by signing the Investigator Responsibilities Document. A copy of this document will be kept on file within the Joint Research Office.

(ii) you, as Principal Investigator, ensuring compliance of the project with all other legislation and guidelines including Caldicott Guardian approvals and compliance with the Data Protection Act 1998, Health and Safety at Work Act 1974, any requirements of the MHRA (*eg* CTA, EudraCT registration), and any other relevant UK/European guidelines or legislation (*eg* reporting of suspected adverse incidents).

(iii) where applicable, you, as Principal Investigator, should also adhere to the GMC supplementary guidance *Good practice in research* and *Consent to research* which sets out the good practice principles that doctors are expected to understand and follow if they are involved in research – see http://www.gmc-uk.org/guidance/ethical_guidance/5991.asp

The NIHR requires NHS organisations to recruit patients to CLRN Portfolio studies within 30 days from the date of this letter. The 30 day deadline for recruiting the first patient is therefore 11 December 2014.

Please note: the Department of Health 70-day bench mark requires recruitment within 70 days of a valid SSI submission. Therefore, recruiting within the NIHR 30 day target will ensure compliance with both targets.

1

Appendix 4. 3: Ethical approval

NHS
Health Research Authority
NRES Committee East Midlands - Northampton
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 883 9440

28 August 2014

Mrs Azizah Mat Hussin
Campus for Ageing and Vitality
Newcastle University
Newcastle Upon Tyne
NE4 5PL

Dear Mrs Mat Hussin,

Study title:	**A cross-sectional study to evaluate the association between Vitamin D status and endothelial function in overweight and obese, post-menopausal women.**
REC reference:	**14/EM/1073**
IRAS project ID:	**152807**

Thank you for your letter of 26 August, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Rebecca Morledge, NRESCommittee.EastMidlands-Northampton@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [Flier recruitment]	2	27 May 2014
Covering letter on headed paper [Cover letter]	1	27 May 2014
GP/consultant information sheets or letters [Result letter to participant]	1	27 May 2014
IRAS Checklist XML [Checklist_03072014]		03 July 2014
Letter from funder [Letter from MARA]		27 May 2014
Letters of invitation to participant [Letters of invitation]	2	27 May 2014
Non-validated questionnaire [Sun exposure questionnaire]	1	29 May 2014
Other [CV John Mathers]	1	27 May 2014
Other [Food frequency questionnaire]		20 June 2014
Other [Email recruitment]	2	27 May 2014
Other [Nitrate intake questionnaire]	1	27 May 2014
Other [Insurance policy]		13 June 2014
Other [CV Tom Hill]		29 May 2014
Other [Leaflet]	2	27 May 2014
Participant consent form [Consent form]	3	27 May 2014
Participant information sheet (PIS)	4	01 August 2014
Participant information sheet (PIS)	3	01 August 2014
REC Application Form [REC_Form_03072014]		03 July 2014
Research protocol or project proposal [Research protocol]	5	31 March 2014
Response to Request for Further Information		05 August 2014
Summary CV for Chief Investigator (CI) [CV Azizah]	1	27 May 2014
Summary CV for student [CV student]	1	27 May 2014
Summary CV for supervisor (student research) [CV Mario]		27 May 2014
Validated questionnaire [IPAQ questionnaire]	1	27 May 2014

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

Appendix 4. 4: Substantial amendment

NHS
Health Research Authority
NRES Committee East Midlands - Northampton
Royal Standard Place
Nottingham
NG1 6FS

Tel: 01158839269

11 August 2015

Mrs Azizah Mat Hussin
Student
Institute for Ageing and Health
Campus for Ageing and Vitality
Newcastle University
Newcastle Upon Tyne
NE4 5PL

Dear Mrs Mat Hussin

Study title:	**A cross-sectional study to evaluate the association between Vitamin D status and endothelial function in overweight and obese, post-menopausal women.**
REC reference:	**14/EM/1073**
Amendment date:	**04 August 2015**
IRAS project ID:	**152807**

Thank you for your letter of 04 August 2015, notifying the Committee of the above amendment.

The Committee does not consider this to be a "substantial amendment" as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Notice of Minor Amendment [To date, have received 78 participants (target 80), study needs to be extended from 31.07.2015 to 31.12.2015. Extra months required to complete recruitment & the analysis of samples (plasma/serum & urine) in the laboratory.]		04 August 2015

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for

Appendix 4. 5: Physical Activity Questionnaire

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

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

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

   _____ **days per week**

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

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

   _____ **hours per day**

   _____ **minutes per day**

   ☐ Don't know/Not sure

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

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

   _____ **days per week**

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

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

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

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

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

_____ **days per week**

☐ No walking → Skip ***to question 7***

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

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

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

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

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

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

Appendix 4. 6: Food Frequency Questionnaire

FFQ Questionnaire

About the food you eat

The following questions are about the food you usually eat and how often you eat certain foods. Please read the following instructions before answering the questions.

For each food there is an amount shown, either a "medium serving" or a common household unit such as a slice or teaspoon. Please put a tick in the box to indicate how often, **on average**, you have eaten the specified amount of each food **during the past year.**

EXAMPLE:

For white bread the amount is one slice, so if you ate 4 or 5 slices a day, you should put a tick in the column headed "4-5 per day".

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
BREAD & SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls								✓	

EXAMPLE:

For chips, the amount is a "medium serving", so if you had a helping of chips twice a week you should put a tick in the column headed "2-4 per week".

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
POTATOES, RICE & PASTA (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chips				✓					

It is your turn to answer now!

Please put a tick in each box to indicate how often, **on average**, you have eaten each food **during the past year.**

Please estimate your average food use as best you can, and please answer every question - do not leave **ANY** lines blank. **Please put a tick on every line.**

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
19. MEAT & FISH *(medium serving)*	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew casserole, curry or bolognese	1	2	3	4	5	6	7	8	9
Beefburgers	1	2	3	4	5	6	7	8	9
Pork: roast, chops, stew, slice or curry	1	2	3	4	5	6	7	8	9
Lamb: roast, chops, stew or curry	1	2	3	4	5	6	7	8	9
Chicken, turkey or other poultry: including fried, casseroles or curry	1	2	3	4	5	6	7	8	9
Bacon	1	2	3	4	5	6	7	8	9
Ham	1	2	3	4	5	6	7	8	9
Corned beef, Spam, luncheon meats	1	2	3	4	5	6	7	8	9
Sausages	1	2	3	4	5	6	7	8	9
Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls, scotch egg	1	2	3	4	5	6	7	8	9
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
PLEASE PUT A TICK ON EVERY LINE.									

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
21. CEREALS *(one bowl)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Porridge, Readybrek	1	2	3	4	5	6	7	8	9
Sugar coated cereals e.g. Sugar Puffs, Cocoa Pops, Frosties	1	2	3	4	5	6	7	8	9
Non-sugar coated cereals e.g. Cornflakes, Rice Crispies	1	2	3	4	5	6	7	8	9
All Bran, Bran Flakes, Muesli	1	2	3	4	5	6	7	8	9
Wholegrain cereals e.g. Cheerios, Weetabix, Shredded Wheat	1	2	3	4	5	6	7	8	9
22. POTATOES, RICE & PASTA *(medium serving)*									
Boiled, mashed, instant or jacket potatoes	1	2	3	4	5	6	7	8	9
Chips, potato waffles	1	2	3	4	5	6	7	8	9
Roast potatoes	1	2	3	4	5	6	7	8	9
Yorkshire pudding, pancakes, dumpling	1	2	3	4	5	6	7	8	9
Potato salad	1	2	3	4	5	6	7	8	9
White rice	1	2	3	4	5	6	7	8	9
Brown rice	1	2	3	4	5	6	7	8	9
White or green pasta, e.g. spaghetti, macaroni, noodles	1	2	3	4	5	6	7	8	9
Tinned pasta, e.g. spaghetti, ravioli, macaroni	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
22. POTATOES, RICE & PASTA (continued) *(medium serving)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Super noodles, pot noodles, pot savouries	1	2	3	4	5	6	7	8	9
Wholemeal pasta	1	2	3	4	5	6	7	8	9
Lasagne, moussaka, cannelloni	1	2	3	4	5	6	7	8	9
Pizza	1	2	3	4	5	6	7	8	9
23. DAIRY PRODUCTS & FATS									
Single or sour cream *(tablespoon)*	1	2	3	4	5	6	7	8	9
Double or clotted cream *(tablespoon)*	1	2	3	4	5	6	7	8	9
Low fat yoghurt, fromage frais *(125g carton)*	1	2	3	4	5	6	7	8	9
Full fat or Greek yoghurt *(125g carton)*	1	2	3	4	5	6	7	8	9
Dairy desserts *(125g carton)*, e.g. mousse	1	2	3	4	5	6	7	8	9
Cheese, e.g. Cheddar, Brie, Edam *(medium serving)*	1	2	3	4	5	6	7	8	9
Cottage cheese, low fat soft cheese *(medium serving)*	1	2	3	4	5	6	7	8	9
Eggs as boiled, fried, scrambled, omelette etc. *(one)*	1	2	3	4	5	6	7	8	9
Quiche *(medium serving)*	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
23.(b) The following on bread or vegetables *(teaspoon)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter	1	2	3	4	5	6	7	8	9
Block margarine, e.g. Stork, Krona	1	2	3	4	5	6	7	8	9
Polyunsaturated margarine, e.g. Flora sunflower	1	2	3	4	5	6	7	8	9
Other soft margarine, dairy spreads, e.g. Blue Band, Clover	1	2	3	4	5	6	7	8	9
Low fat spread, e.g. Gold	1	2	3	4	5	6	7	8	9
24. SWEETS & SNACKS									
Sweet biscuits, chocolate, e.g. digestive *(one)*	1	2	3	4	5	6	7	8	9
Sweet biscuits, plain, e.g. Nice, ginger *(one)*	1	2	3	4	5	6	7	8	9
Cakes e.g. fruit, sponge, sponge pudding *(medium serving)*	1	2	3	4	5	6	7	8	9
Sweet buns & pastries e.g. flapjacks, doughnuts, Danish pastries, cream cakes *(medium serving)*	1	2	3	4	5	6	7	8	9
Fruit pies, tarts, crumbles *(medium serving)*	1	2	3	4	5	6	7	8	9
Milk puddings, e.g. rice, custard, trifle *(medium serving)*	1	2	3	4	5	6	7	8	9
Chocolates *(small bar or ¼ pound of chocolates)*	1	2	3	4	5	6	7	8	9
Ice cream, choc ices *(one)*	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
24. SWEETS & SNACKS (continued)	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweets, toffees, mints *(one packet)*	1	2	3	4	5	6	7	8	9
Sugar added to tea, coffee, cereal *(teaspoon)*	1	2	3	4	5	6	7	8	9
Crisps or other packet snacks e.g. Wotsits *(one packet)*	1	2	3	4	5	6	7	8	9
Peanuts or other nuts *(one packet)*	1	2	3	4	5	6	7	8	9
25. SOUPS, SAUCES AND SPREADS									
Vegetable soups *(bowl)*	1	2	3	4	5	6	7	8	9
Meat soups *(bowl)*	1	2	3	4	5	6	7	8	9
Sauces, e.g. white sauce, cheese sauce, gravy *(medium serving)*	1	2	3	4	5	6	7	8	9
Tomato based sauces, e.g. pasta sauces *(medium serving)*	1	2	3	4	5	6	7	8	9
Tomato ketchup, brown sauce *(tablespoon)*	1	2	3	4	5	6	7	8	9
Relishes, e. g. pickles, chutney, mustard *(tablespoon)*	1	2	3	4	5	6	7	8	9
Low calorie, low fat salad cream or mayonnaise *(tablespoon)*	1	2	3	4	5	6	7	8	9
Salad cream, mayonnaise *(tablespoon)*	1	2	3	4	5	6	7	8	9
French dressing *(tablespoon)*	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
26. DRINKS (continued)	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Spirits, e.g. gin, brandy, whisky, vodka *(single)*	1	2	3	4	5	6	7	8	9
Low calorie or diet fizzy soft drinks *(glass)*	1	2	3	4	5	6	7	8	9
Fizzy soft drinks, e.g. Coca cola, lemonade *(glass)*	1	2	3	4	5	6	7	8	9
Pure fruit juice (100%), e.g. orange, apple juice *(glass)*	1	2	3	4	5	6	7	8	9
Fruit squash or cordial *(glass)*	1	2	3	4	5	6	7	8	9
27. FRUIT *(1 fruit or medium serving)* *For very seasonal fruits such as strawberries, please estimate your average use when the fruit is in season									
Apples	1	2	3	4	5	6	7	8	9
Pears	1	2	3	4	5	6	7	8	9
Oranges, satsumas, mandarins, tangerines, clementines	1	2	3	4	5	6	7	8	9
Grapefruit	1	2	3	4	5	6	7	8	9
Bananas	1	2	3	4	5	6	7	8	9
Grapes	1	2	3	4	5	6	7	8	9
Melon	1	2	3	4	5	6	7	8	9
*Peaches, plums, apricots, nectarines	1	2	3	4	5	6	7	8	9
*Strawberries, raspberries, kiwi fruit	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
28. VEGETABLES Fresh, frozen or tinned (continued) ***(medium serving)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Mushrooms	1	2	3	4	5	6	7	8	9
Sweet peppers	1	2	3	4	5	6	7	8	9
Beansprouts	1	2	3	4	5	6	7	8	9
Green salad, lettuce, cucumber, celery	1	2	3	4	5	6	7	8	9
Mixed vegetables (frozen or tinned)	1	2	3	4	5	6	7	8	9
Watercress	1	2	3	4	5	6	7	8	9
Tomatoes	1	2	3	4	5	6	7	8	9
Sweetcorn	1	2	3	4	5	6	7	8	9
Beetroot, radishes	1	2	3	4	5	6	7	8	9
Coleslaw	1	2	3	4	5	6	7	8	9
Avocado	1	2	3	4	5	6	7	8	9
Baked Beans	1	2	3	4	5	6	7	8	9
Dried lentils, beans, peas	1	2	3	4	5	6	7	8	9
Tofu, soya meat (TVP), Vegeburger	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

YOUR DIET LAST YEAR, continued

29. (a) What type of milk did you most often use?

Select one only

Full cream.....❒1
Channel Islands.....❒2
Dried milk.....❒3
Semi-skimmed.....❒4
Skimmed.....❒5
Soya.....❒6
Other.....❒7
None.....❒8

4/52

29. (b) Approximately, how much milk did you drink each day, including milk with tea, coffee, cereals etc?

None.....❒1
Quarter of a pint (roughly 125mls).....❒2
Half a pint (roughly 250mls) .....❒3
Three quarters of a pint (roughly 375mls) .....❒4
One pint (roughly 500mls) .....❒5
More than one pint (more than 500mls) .....❒6

4/53

30. What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter.....❒1
Lard/dripping.....❒2
Solid vegetable fat.....❒3
Margarine.....❒4
Vegetable oil.....❒5
Olive oil.....❒6
None.....❒7

4/54

31. How often did you eat food that was fried at home?

Select one only

Daily.....❒1
1-3 times a week.....❒2
4-6 times a week.....❒3
Less than once a week.....❒4
Never.....❒5

4/55

32. How often did you eat fried food *away* from home?

Select one only

Daily.....❒1
1-3 times a week.....❒2
4-6 times a week.....❒3
Less than once a week.....❒4
Never.....❒5

4/56

33. (a) How often did you add salt to food while cooking?

Select one only

Always.....❒1
Usually.....❒2
Sometimes.....❒3
Rarely.....❒4
Never.....❒5

4/57

33. (b) How often did you add salt to any food at the table?

Select one only

Always.....❒1
Usually.....❒2
Sometimes.....❒3
Rarely.....❒4
Never.....❒5

4/58

34. Do you follow a special diet?
Please tick all that apply.

No.....❒1
Yes, due to a medical condition/allergy.....❒2
Yes, to lose weight.....❒3
Yes, because of personal beliefs .....❒4
Yes other.....❒5

4/63

35. Over the last year, how often have you eaten organic foods?

Select one only.

Most days.....❒1
Once or twice a week.....❒2
Once a month.....❒3
Never/hardly ever.....❒4

4/64

36. Have you taken any of the following during the past year?

a) Vitamins (e.g. multivitamins, vitamin B, vitamin C, folic acid)

Yes.....❒1
No.....❒2

If YES, what type and when _____

4/65

b) Minerals (e.g. iron, calcium, zinc, magnesium)

Yes.....❒1
No.....❒2

If YES, what type and when _____

4/66

c) Fish oils (e.g. cod liver oil, omega-3)

Yes.....❒1
No.....❒2

If YES, what type and when _____

4/67

d) Other food supplements (e.g. oil of evening primose, starflower oil, royal jelly, ginseng)

Yes.....❒1
No.....❒2

If so, what type and when _____

4/68

37. During the course of last year, on average, how many times did you eat the following foods?

Food Type	Times/week	Portion size
Vegetables (not including potatoes)		medium serving 5/41
Salads		medium serving 5/43
Fruit and fruit products (not including fruit juice)		medium serving or 1 fruit 5/45
Fish and fish products		medium serving 5/47
Meat, meat products and meat dishes (including bacon, ham and chicken)		medium serving 5/49

38. How often do you eat fruit or vegetables from a garden or allotment?

Select one only.

Most days.....❒1
Once or twice a week.....❒2
Once a month.....❒3
Never/hardly ever.....❒4

5/50

39. How would you most commonly eat the following vegetables? If you have not eaten the vegetable listed within the last year, please tick the 'Not eaten ' box.

Select one box only for each vegetable;

	Raw	Boiled < 10mins	Boiled >10mins	Steamed	Fried	Not eaten
Asparagus	❒	❒	❒	❒	❒	❒
Artichoke	❒	❒	❒	❒	❒	❒
Beansprouts	❒	❒	❒	❒	❒	❒
Beetroot	❒	❒	❒	❒	❒	❒
Broad beans	❒	❒	❒	❒	❒	❒
Broccoli	❒	❒	❒	❒	❒	❒
Brussel Sprouts	❒	❒	❒	❒	❒	❒
Cauliflower	❒	❒	❒	❒	❒	❒

	Raw	Boiled < 10mins	Boiled >10mins	Steamed	Fried	Not eaten
Cabbage	❒	❒	❒	❒	❒	❒
Chick Peas	❒	❒	❒	❒	❒	❒
Courgette	❒	❒	❒	❒	❒	❒
Curly Kale	❒	❒	❒	❒	❒	❒
Green Beans	❒	❒	❒	❒	❒	❒
Leeks	❒	❒	❒	❒	❒	❒
Lentils	❒	❒	❒	❒	❒	❒
Lettuce	❒	❒	❒	❒	❒	❒
Mixed veg frozen	❒	❒	❒	❒	❒	❒
Mixed veg canned	❒	❒	❒	❒	❒	❒
Parsnip	❒	❒	❒	❒	❒	❒
Peas	❒	❒	❒	❒	❒	❒
Red Kidney Beans	❒	❒	❒	❒	❒	❒
Runner Beans	❒	❒	❒	❒	❒	❒
Spinach fresh	❒	❒	❒	❒	❒	❒
Spinach frozen	❒	❒	❒	❒	❒	❒
Sweetcorn fresh	❒	❒	❒	❒	❒	❒
Sweetcorn canned	❒	❒	❒	❒	❒	❒

39. How would you most commonly eat the following vegetables? If you have not eaten the vegetable listed within the last year, please tick the 'Not eaten ' box.

Select one box only for each vegetable;

	Raw	Boiled < 10mins	Boiled >10mins	Steamed	Fried	Not eaten
Asparagus	❒	❒	❒	❒	❒	❒
Artichoke	❒	❒	❒	❒	❒	❒
Beansprouts	❒	❒	❒	❒	❒	❒
Beetroot	❒	❒	❒	❒	❒	❒
Broad beans	❒	❒	❒	❒	❒	❒
Broccoli	❒	❒	❒	❒	❒	❒
Brussel Sprouts	❒	❒	❒	❒	❒	❒
Cauliflower	❒	❒	❒	❒	❒	❒

	Raw	Boiled < 10mins	Boiled >10mins	Steamed	Fried	Not eaten
Cabbage	❒	❒	❒	❒	❒	❒
Chick Peas	❒	❒	❒	❒	❒	❒
Courgette	❒	❒	❒	❒	❒	❒
Curly Kale	❒	❒	❒	❒	❒	❒
Green Beans	❒	❒	❒	❒	❒	❒
Leeks	❒	❒	❒	❒	❒	❒
Lentils	❒	❒	❒	❒	❒	❒
Lettuce	❒	❒	❒	❒	❒	❒
Mixed veg frozen	❒	❒	❒	❒	❒	❒
Mixed veg canned	❒	❒	❒	❒	❒	❒
Parsnip	❒	❒	❒	❒	❒	❒
Peas	❒	❒	❒	❒	❒	❒
Red Kidney Beans	❒	❒	❒	❒	❒	❒
Runner Beans	❒	❒	❒	❒	❒	❒
Spinach fresh	❒	❒	❒	❒	❒	❒
Spinach frozen	❒	❒	❒	❒	❒	❒
Sweetcorn fresh	❒	❒	❒	❒	❒	❒
Sweetcorn canned	❒	❒	❒	❒	❒	❒

40. **For the following foods, please list the top three makes and/or brands you most commonly consume.**
If you do not eat this type of food please tick the 'not eaten' box.

Bread: 1:_____

2:_____

3:_____ Not eaten ❒

Breakfast Cereal

1:_____

2:_____

3:_____ Not eaten ❒

About you and your health

41. **For your age, would you say that your health was:**

Please tick one box on the scale of 1 to 5:

	1	2	3	4	5	
very good	❒	❒	❒	❒	❒	very poor

5/51

42. **Which of the following best describes your daily work or other daytime activity that you usually do?**

Please tick one box only.

I am usually sitting and do not walk about much.....❒1

I stand or walk about quite a lot, but do not have to carry or lift things very often .....❒2

I usually lift or carry light loads or have to climb stairs or hills often .....❒3

I do heavy work or carry heavy loads often .....❒4

5/52

43. **Please give the average number of hours per week you spend doing sports and other activities.**

Please write in the amount for each; if none write "0"

a) Mildly energetic

(e.g. walking, gardening, playing darts, general housework) _____ hour/s

b) Moderately energetic

(e.g. heavy housework or gardening, dancing, golf, cycling, leisurely swimming) _____ hour/s

c) Vigorous

(e.g. running, competitive swimming or cycling, tennis, football, squash, aerobics) _____ hour/s

5/64

44. Do you smoke?

Yes, I smoke daily .....❒1
Yes, I smoke occasionally .....❒2
No, I used to smoke .....❒3
No, I have never smoked .....❒4

5/65

44. If yes or you used to smoke, how much, on average, do you (or did you) smoke a day?
Please write in the amount for each; if none write "0"

Cigarettes _____
Cigars _____
Ounces tobacco _____

5/74

45. In the past 12 months have you taken an alcoholic drink:
Please tick one box.

Twice a day or more .....❒1
Almost daily .....❒2
Once or twice a week .....❒3
Once or twice a month .....❒4
Special occasions only .....❒5
Not at all .....❒6

5/75

46. In a typical 7-day week, including the weekend, how many standard drinks of alcohol do you drink? *(see the table below)*
Please write the number in the box below.

I usually drink _____ standard drinks of alcohol per week

5/76

ONE STANDARD DRINK = ½ pint of beer
or ½ pint cider
or ½ pint lager
or 1 glass of wine, martini, or cinzano
or 1 small glass of Sherry or Port
or 1 measure of Spirits (gin, whiskey, vodka etc.)
or 1 measure liquor

A PINT OF BEER, CIDER, OR LAGER COUNTS AS TWO STANDARD DRINKS
A DOUBLE MEASURE OF SPIRITS COUNTS AS TWO STANDARD DRINKS

47. (a) Do you have any long-term illness, physical or mental health problem or handicap?

Yes.....❒1
No.....❒2

5/91

48. (b) If yes, does this limit your daily activity in any way?

Yes.....❒1
No.....❒2

5/92

THANK YOU FOR TAKING TIME TO COMPLETE THIS QUESTIONNAIRE

Appendix 4. 7: Sunshine Exposure Questionnaire

Sunshine Exposure Questionnaire

This section will help me to understand your personal **sun exposure habits while in the UK.**

1. How much time would you spend outdoors on a **weekday** during the sunny months?
   - ◯Less than 15 minutes
   - ◯Between 15 and 30 minutes
   - ◯Between 30 minutes and 1 hour
   - ◯Between 1 hour and 2 hours
   - ◯More than 2 hours

2. How much time would you spend outdoors on a **weekend** day during the sunny months?
   - ◯Less than 15 minutes
   - ◯Between 15 and 30 minutes
   - ◯Between 30 minutes and 1 hour
   - ◯Between 1 hour and 2 hours
   - ◯More than 2 hours

3. When you are outdoors during the sunny months, do you mostly wear…

(**List 1-4 in order of priority, no. 1 being the one you would do the most and no. 4 being the one you would do the least**)

	Order of Priority
Long sleeves and **long** trousers or stockings	
Short sleeves and **long** trousers/skirt	
Short Sleeves and **Short** skirt/shorts	
Swimwear or light beach clothes	

4. Do you veil, and if so to what extent of your body is covered?

   - ◯ Not applicable (I do not veil)
   - ◯ Fully covered (eyes exposed)
   - ◯ Partially covered

5. During the sunny months, how often would you apply sunscreen? (In the UK- not on holiday)

   Please tick or circle your answer
   Never Rarely Sometimes Usually Always Other

6. What sun protection factor do you usually apply? _____

7. What environment does your occupation expose you to?

   - ◯Indoors
   - ◯Outdoors
   - ◯Mixture

Appendix 5. 1: Ethics Approval

Faculty of Medical Sciences
Newcastle University
The Medical School
Framlington Place
Newcastle upon Tyne
NE2 4HH United Kingdom

Dr Mario Siervo
Institute of Cellular Medicine

FACULTY OF MEDICAL SCIENCES: ETHICS COMMITTEE

Dear Mario,

Title: Ageing, Vitamin D and Nitric Oxide Production in Healthy Participants
Application No: 1285/15004/2017
Start date to end date: 19/06/2017 to 30/09/2017

On behalf of the Faculty of Medical Sciences Ethics Committee, I am writing to confirm that the ethical aspects of your proposal have been considered and your study has been given ethical approval.

The approval is limited to this project: **1285/15004/2017**. If you wish for a further approval to extend this project, please submit a re-application to the FMS Ethics Committee and this will be considered.

During the course of your research project you may find it necessary to revise your protocol. Substantial changes in methodology, or changes that impact on the interface between the researcher and the participants must be considered by the FMS Ethics Committee, prior to implementation.*

At the close of your research project, please report any adverse events that have occurred and the actions that were taken to the FMS Ethics Committee.*

Best wishes,

Yours sincerely

Kimberley Sutherland
On behalf of Faculty Ethics Committee

cc.
Professor Daniel Nettle, Chair of FMS Ethics Committee
Mrs Kay Howes, Research Manager

*Please refer to the latest guidance available on the internal Newcastle web-site.

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Appendix 5. 2: Standarized low NO_3^- Diet

Standardized low NO_3^- Diet

In order to increase the precision and validity of our measurements during the study, we need to control the amount of nitrate in your diet and you should try to follow the dietary guidelines provided below. The dietary plan will be thoroughly explained to you and if you have any other questions about the diet during the study please let us know and we will be very happy to clarify them to you.

A list of food allowed and food to avoid is shown below.

Food to avoid	Food allowed		
• Rocket • Beet • Beetroot • Broccoli • Brussels sprouts • Cabbage • Canned asparagus • Canned beans • Canned corn • Canned peas • Canned pumpkin • Canned spinach • Canned tomatoes • Cauliflower • Celery • Chard • Chicory • Chinese Leaf • Chives • Coriander • Cured beef • Dill • Endive • Fennel • Ham • Kale	• Apples • Apricots • Artichoke • Asparagus • Avocados • Bacon • Bananas • Basil • Beans • Beans (French) • Beans (Runner) • Blackberries • Barley • Beans (Broad) • Beansprout • Blueberries • Blue Cheese • Bread • Butter • Camembert • Cantaloupe • Cereal • Cheese Soft • Cheese Spread • Cherries • Chicken • Corn • Corn oil	• Cured Turkey • Currants • Dried Beef • Frankfurt • Hot peppers • Egg • Eggplant • Fresh Pepper • Feta Cheese • Flour • Fresh Cheese • Fresh Sausage • Fresh Tomato • Grapefruit • Garlic • Grapes • Ketchup • Kiwi • Mayonnaise • Meat Fresh • Milk • Minced Meat • Mushrooms • Mandarin Oranges • Mango • Melon • Mustard and Cress • Onion (Spring)	• Pumpkin • Parsnip • Pasta • Peach • Peas • Pineapples • Plums • Potatoes • Poultry • Prickly Pears • Radish • Raspberries • Red and yellow peppers • Quinces • Strawberries, blueberries • String beans • Summer squash • Sunflower oil • Sweet almond oil • Rice • Ricotta • Soybean oil • Sweet corn • Sweet potato • Watermelon • Wheat • Wheat flour

Food to avoid	Food allowed
• Green Beans • Leek • Lettuce • Meat Cured • Rhubarb • Spinach • Turnip • Watercress	• Courgette • Cucumber • Cured Pork • Cured Salami • Peanuts • White bread • Winter melon • Wheat germ oil • Yogurt

Appendix 5. 3: Saliva collection procedures

Day 1

5.00pm
- 1st collection of saliva sample
- Drink the sodium nitrate solution
- Blood pressure measurement

7.00pm
- Have snacks and drink (10 minutes)

7.30pm
- 2nd collection of saliva sample
- Blood pressure measurement

8.15pm
- 3rd collection of saliva sample
- Blood pressure measurement

9.00pm
- 4th collection of saliva sample
- Blood pressure measurement

10.00pm
- 5th collection of saliva sample
- Blood pressure measurement

Day 2

7.00am
- 6th collection of saliva sample
- Blood pressure measurement

8.00am
- 7th collection of saliva sample
- Blood pressure measurement

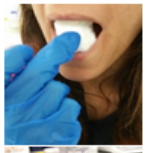
9.00am
- 8th collection of saliva sample
- Blood pressure measurement

Saliva collection procedures

1. Avoid to drink water 20 minutes before the measurements

2. Put a glove or just be sure to have clean hands

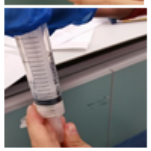
3. Put a cotton ball in your mouth
   Chew for 2 minutes or until you
   feel the cotton ball is soaked with saliva



4. Remove the plunger from the barrel of the syringe

5.Put the cotton ball in the syringe

6. Press the cotton ball with the plunger
   and collect the saliva in the small tube



7. Close the lid of the tube and put it in the box

Frequent asked questions

1) Can I eat during the study duration?

Answer: You are not allowed to eat **4 hours** before the 1st saliva sample collection (5.00 pm). However, you can eat the snack that we provide you at 7.00pm (please refer details on the next page). You can have you breakfast the next morning after your last saliva sample collection (9 am).

2) Can I drink during the study duration?

Answer: You can drink the water that we provide you (Buxton water in bottle). You can drink whenever you want but avoid drinking water **20 minutes** before the saliva sample collection.

3) Can I brush my teeth or use the mouth wash?

Answer: Yes, you can but avoid doing it **20 minutes** before the saliva sample collection.

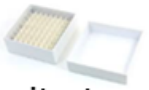
4) Where should I keep the saliva sample?

Answer: The saliva sample should be kept in the **white storage box** that we provide you, in the **freezer.**

5) What if I wake up early the next morning e.g. 6 am. Should I wait for 7 am to collect the saliva sample?

Answer: You can get the saliva sample after you wake up. It is not necessary that you have to start the saliva collection at 7 am, but you have to state the time you take the sample in the form we provide. And the following saliva samples should be after one hour.

Materials provided for the saliva sample collection

- Syringe – 8 items  X 8
- Small tube (Eppendorf tube)– 8 items  X 8
- Cotton balls – A small pack 
- Gloves 
- White storage box 
- A bottle of sodium nitrate solution 

Appendix 5. 4: Participant's checklist

Participant's checklist

No	Task	Remarks
	Day 1	
1.	5.00pm • 1st collection of saliva sample ☐ • Drink the sodium nitrate solution ☐ • Blood pressure measurement ☐ **Blood pressure reading** SBP: DBP:	
2.	7.00pm – 7.10pm • Have snacks and drink ☐	
2.	7.30pm • 2nd collection of saliva sample ☐ • Blood pressure measurement ☐ **Blood pressure reading** SBP: DBP:	
3.	8.15pm • 3rd collection of saliva sample ☐ • Blood pressure measurement ☐ **Blood pressure reading** SBP: DBP:	
4.	9.00pm • 4th collection of saliva sample ☐ • Blood pressure measurement ☐ **Blood pressure reading** SBP: DBP:	
5.	10.00pm • 5th collection of saliva sample ☐ • Blood pressure measurement ☐ **Blood pressure reading** SBP: DBP:	:

	Day 2	
6.	7.00am or specify time: _____am • 6th collection of saliva sample ☐ • Blood pressure measurement ☐ **Blood pressure reading** SBP: DBP:	
7.	8.00am or specify time: _____am • 7th collection of saliva sample ☐ • Blood pressure measurement ☐ **Blood pressure reading** SBP: DBP:	
8.	9.00am or specify time: _____am • 8th collection of saliva sample ☐ • Blood pressure measurement ☐ **Blood pressure reading** SBP: DBP:	
9.	**Questionnaires** 1. Physical activity questionnaire (IPAQ) ☐ 2. Food Frequency questionnaire (FFQ) ☐ 3. Sun exposure questionnaire ☐ 4. Nitrate intake questionnaire ☐	

Appendix 5. 5: Examples of nitrate/nitrite calibration curves and examples of plasma nitrate/nitrite and urinary nitrate analysis

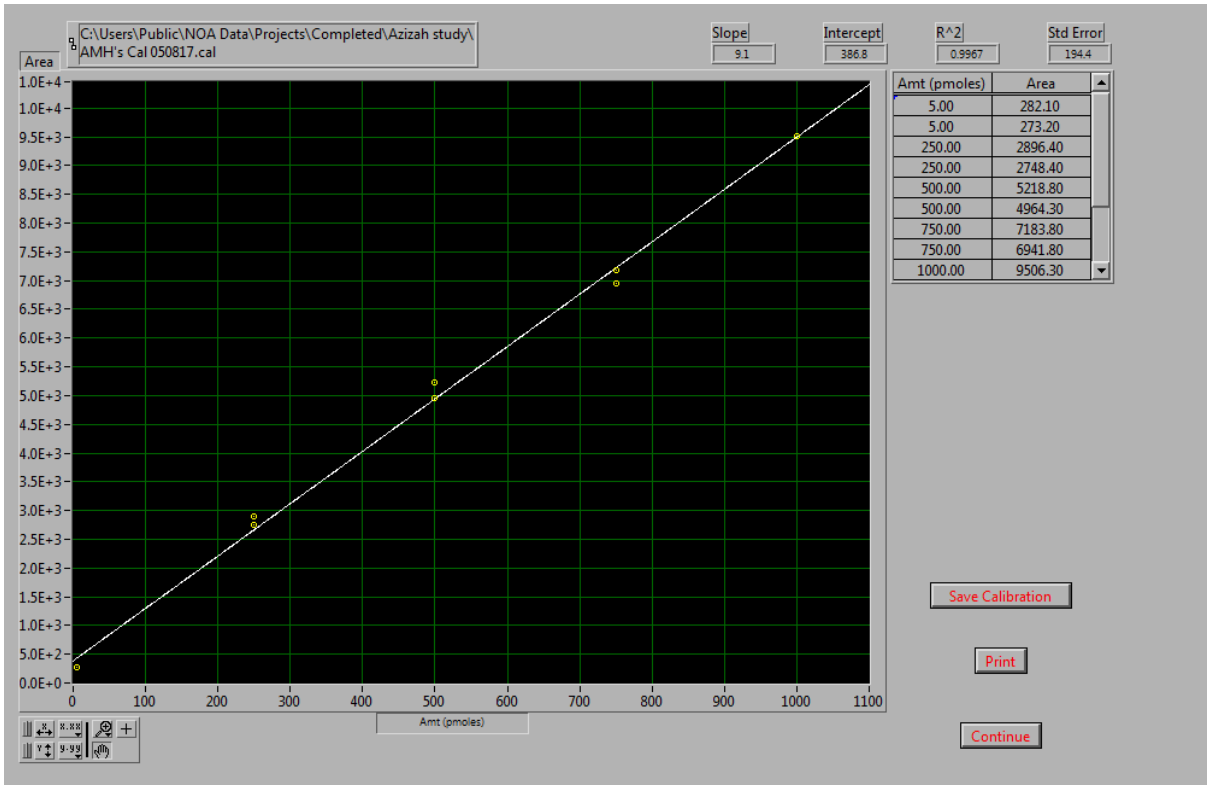


Figure 5.5 A: Examples of nitrate calibration curves.

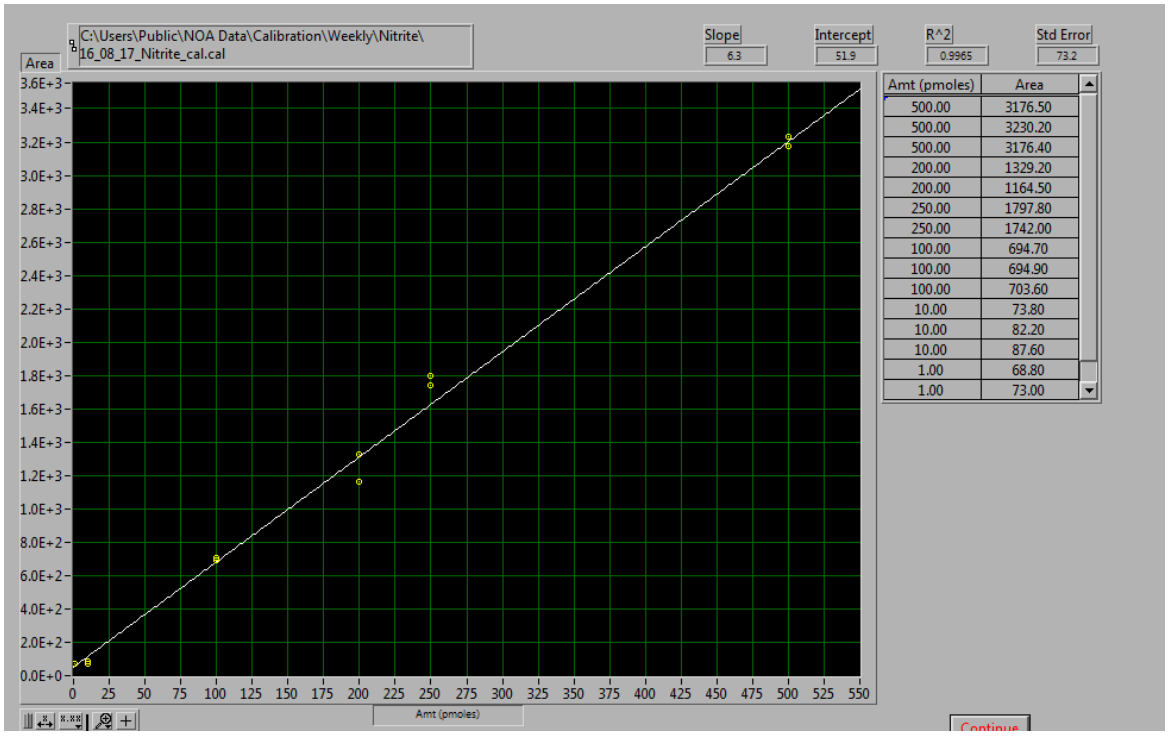


Figure 5.5 B: Examples of nitrite calibration curves.

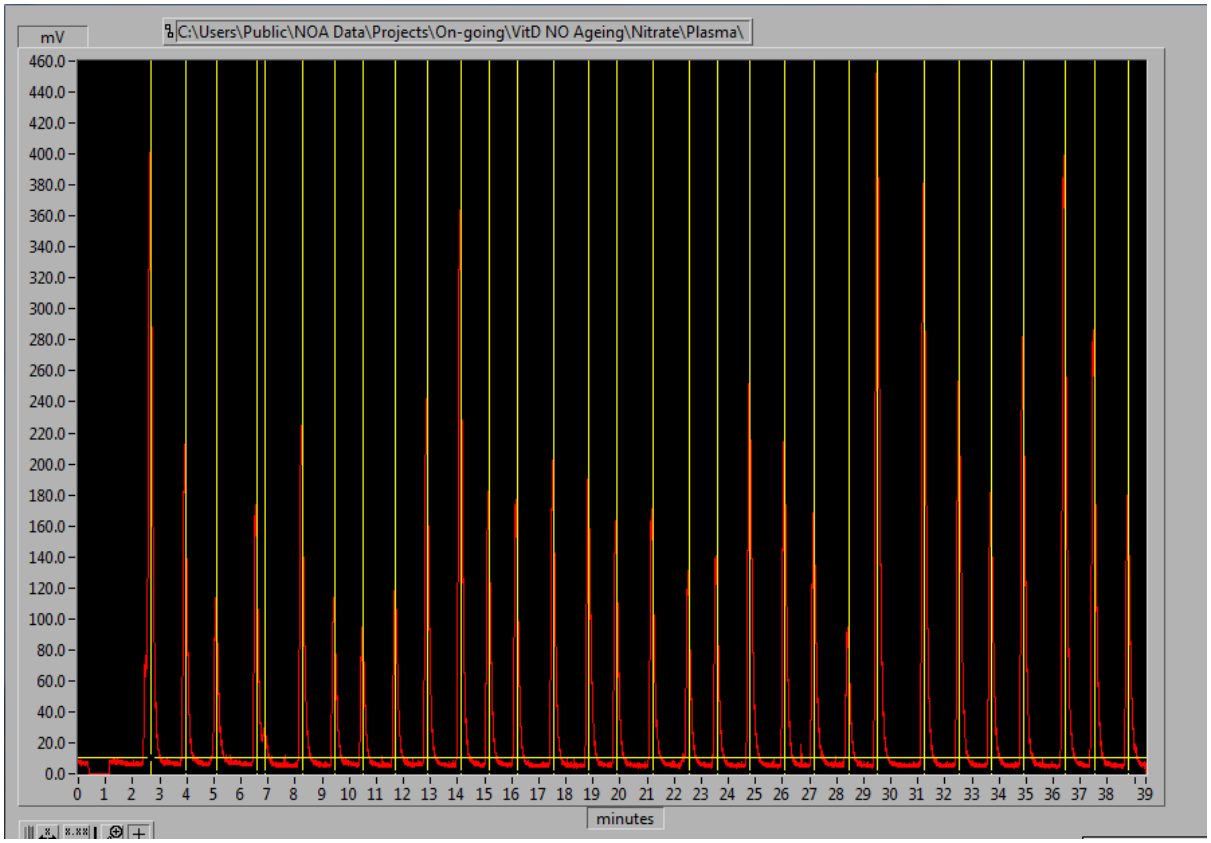


Figure 5.5 C: Examples of plasma nitrate sample analysis

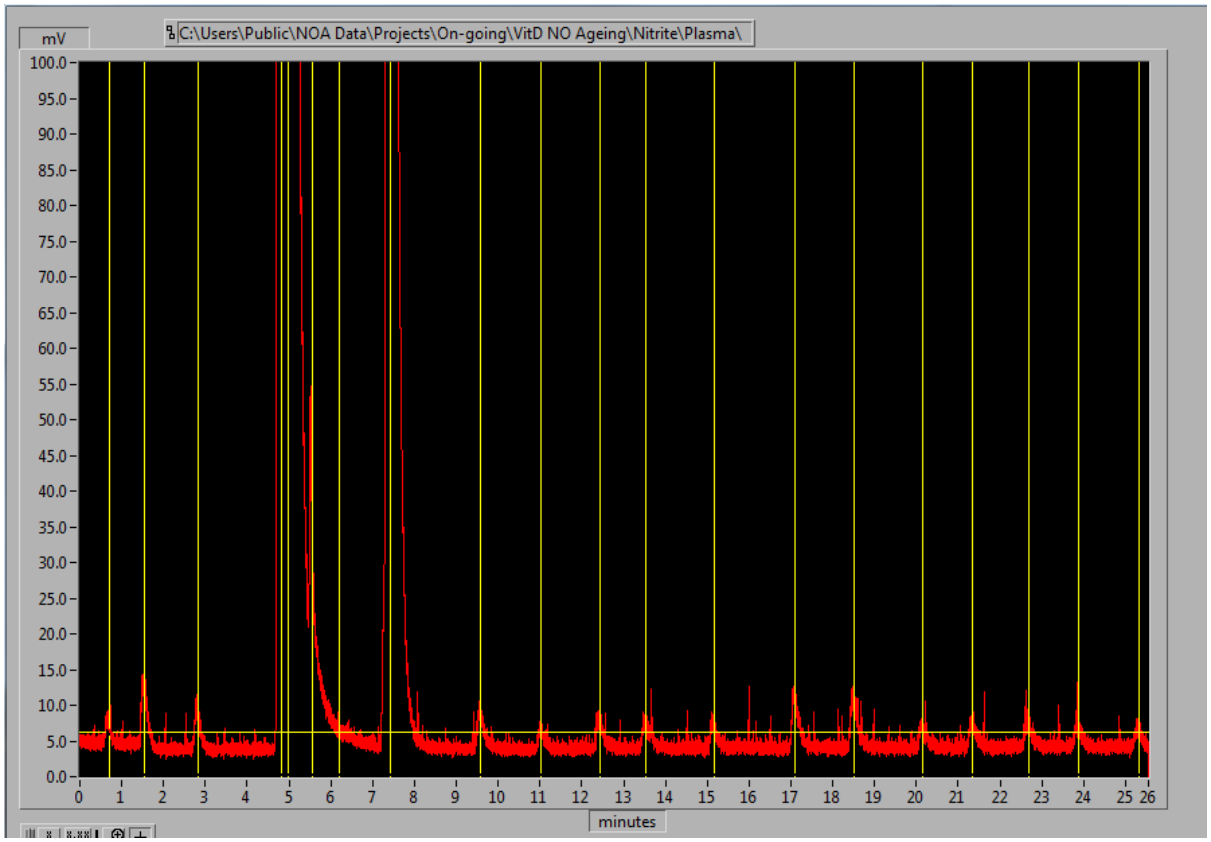


Figure 5.5 D: Examples of plasma nitrite sample analysis

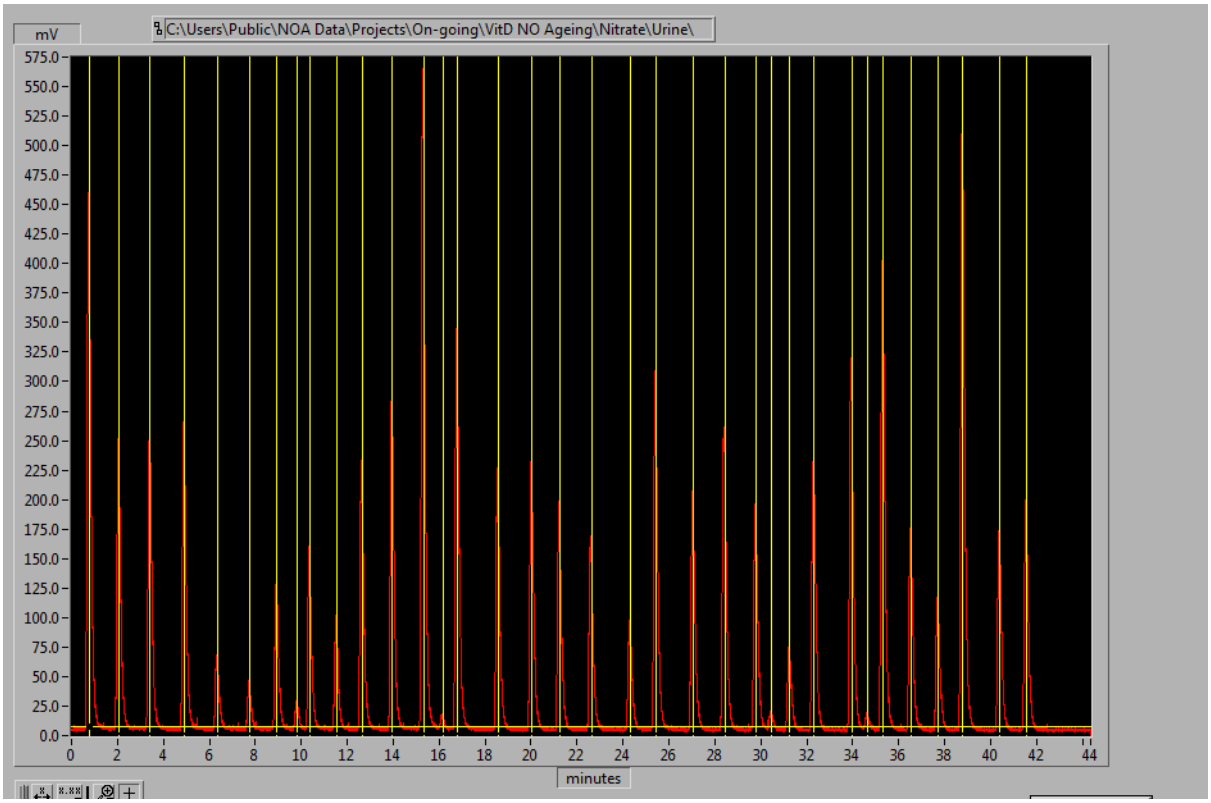


Figure 5.5 E: Examples of urinary nitrate sample analysis

Appendix 5. 6: Performing 25(OH)D analysis

The measurement of 25(OH)D concentrations in this PhD project was done using a commercial kit (25-Hydroxy Vitamin D^{s} Enzyme Immunoassay (EIA) from Immunodiagnosticsystems (IDS) AC-57SF1) in a 96-well format. The following components were required to perform the analyses: 1) Calibrators; 2) Controls; 3) Antibody coated plate; 4) 25-D Biotin concentrate; 5) Enzyme conjugate; 6) TMB substrate; 7) Stop solution 8) and wash concentrate.

Reagent preparation

The serum samples were collected in plain vacutainer tube. All reagents were brought to the room temperature (18 – 25^{o}c) at least 90 minutes before use. The reagents, samples and standards were prepared as instructed;

25-D Biotin solution (supplied in lyophilised condition)

i. 3ml of buffer was added to the bottle of lyophilised 25-D Concentrate and stand for 10-15 minutes at room temperature. The solution was inverted several times to ensure complete reconstitution.
ii. The reconstituted 25-D Biotin Concentrate was added back into the bottle containing the remaining buffer and mixed well.

Wash solution

i. The content of the bottle of Wash Concentrate was added to 950 mL of distilled water and mixed.

Assay procedure

6) Borosilicate glass were labelled, one for each Calibrator, Control and Sample.
7) 25µL of each Calibrator, Control or samples were added to the appropriately labelled tubes.
8) 1 ml of 25-D Biotin Solution was added to all tubes.
9) Each of the tubes was vortexed thoroughly for 10 seconds.
10) 200µL of each diluted calibrator, Control or Sample were added in duplicate to the appropriate well. The plate was covered with the adhesive plate sealer and incubated at room temperature (18c -25c) for 2 hours.

11) The plate was aspirated and washed. This process was repeated for two times for a total of three washes. Each well was filled with wash buffer (250µL) using manifold dispenser. After the last wash, any remaining wash buffer was removed by aspiring or decanting. The plate was inverted and blotted against a clean paper towels.
12) Using multichannel pipette, 200µL of Enzyme Conjugate were added to each of the wells. The plate was covered with a new adhesive strip and incubated for 30 minutes at room temperature.
13) The aspiration/wash process in step 6 was repeated. Using multichannel pipette, 200µL of substrate was added to each well. The plate was covered with a new adhesive strip and incubated for 30 minutes at room temperature.
14) After 30 minutes, by using multichannel pipette, 100µL of stop solution was added to each well.
15) The absorbance in each well was determined within 30 minutes using a microplate reader set to 450 nm.

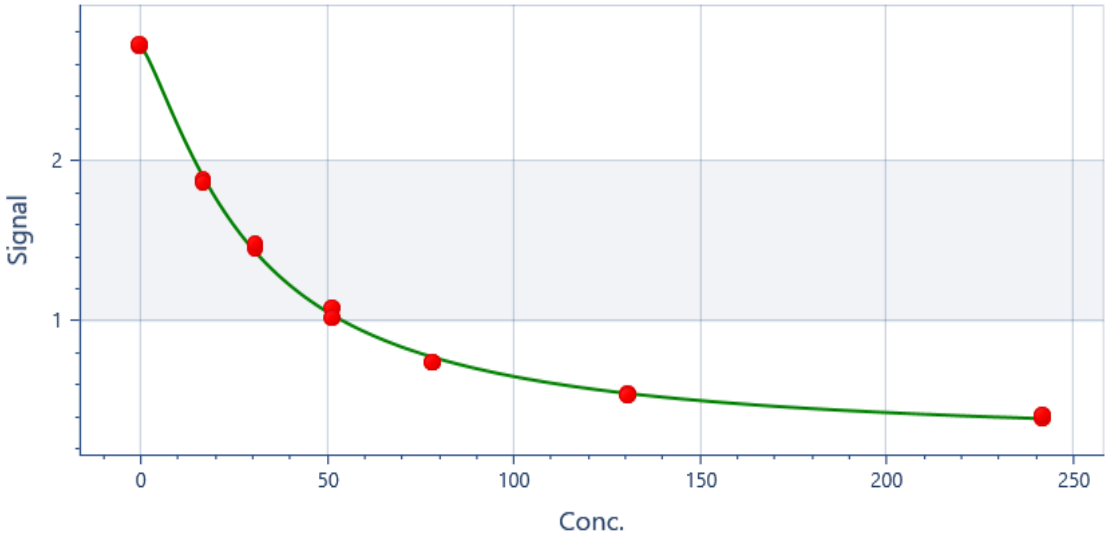


Figure 5.5A: Standard curve 25(OH)D

Appendix 5. 7: Participant's evaluation form

Evaluation Form

At the end of the study on Ageing, Vitamin D and NO production, each participant was asked to complete an anonymous evaluation form to provide feedback on some aspects of the study protocols. Seventeen volunteers out of forty provided their feedback and their answers are reported below.

Question 1: What do you like about the AVIDNO Study?

A1: Checking my blood pressure. Might buy a blood pressure monitor. The researchers were very friendly.
A2: The participant's information sheets were very comprehensive.
A3: It was easy to do at home.
A4: Checking my blood pressure discovering very high.
A5: Well organised and easy instructions.
A6: Like contributing to the future.
A7: The potential outcome.
A8: Instructions were very comprehensive and checklist was helpful.
A9: The information and rationale provided.
A10: Measure my blood pressure and heart rate.
A11: The relaxed way it's carried out.
A12: Study protocol.
A13: Its precision.
A14: I have the results from my healthcare.
A15: Being checked and advised about healthy lifestyle.
A16: I find it very interesting.
A17: No comment

Question 2: Is there anything that you disliked about the AVIDNO Study?

A1: No
A2: No
A3: The first cotton ball to chew.
A4: None
A5: Cotton ball chewing.
A6: Have a limited dinner.
A7: The blood monitor was difficult to set to work.
A8: Not really.
A9: No
A10: No
A11: No
A12: None
A13: No
A14: No
A15: No
A16: No
A17: No

Question 3: If a similar study was conducted in the future, would you be likely to participate?

A1: Yes, if I am still in Newcastle
A2: Yes
A3: Yes

A4: Yes
A5: Yes
A6: Not really sure at this moment.
A7: Yes
A8: Yes
A9: Yes
A10: Yes
A11: Yes
A12: Yes
A13: Yes
A14: Yes
A15: Yes
A16: Yes
A17: Yes

Question 4: Would you add any additional tests or measurements to the ones we took as part of this study?

A1: No
A2: No
A3: Not qualified to suggest any.
A4: Provide extra cotton balls.
A5: Do not know
A6: Not sure
A7: No
A8: No
A9: No
A10: No
A11: I think it would be better to do it over two days
A12: No
A13: No
A14: No
A15: No
A16: No
A17: No

Question 5: What do you think is an appropriate length of time for a study visit?

A1: 1 hour
A2: 2 hours
A3: 1 hour
A4: 1 hour
A5: Less than 2 hours is ok.
A6: About 1 hour
A7: 1 hour and half
A8: 1 hour
A9: 2 hours
A10: Whatever is necessary
A11: 1 hour
A12: 90 minutes
A13: Just nice
A14: 1 and half hour
A15: There is no time limit

A16: No comment
A17: 1 hour

Question 6: What is the maximum time you would be willing to attend a study visit?

A1: 2 hour
A2: 4 hours
A3: 2 hour
A4: 1 hour
A5: No limit.
A6: Whatever was needed
A7: 1 hour and half
A8: 1 hour
A9: 2 hours
A10: As long as necessary
A11: 1 hour
A12: 90 minutes
A13: Just nice
A14: 1 and half hour
A15: There is no time limit
A16: Depends what the study was
A17: 1 hour

Question 7: Are there any improvements that could be made to the study?

A1: No
A2: No
A3: Not that I am aware of
A4: None
A5: Not enough information to comment.
A6: No
A7: Feedback on results.
A8: Not sure
A9: No
A10: No
A11: No
A12: No
A13: No comments
A14: No
A15: No
A16: No
A17: No

Question 8: Do you think the incentive we offer to the participants is sufficient?

A1: Yes
A2: Yes
A3: Yes, generous
A4: Yes
A5: Yes
A6: Yes
A7: Yes
A8: Yes
A9: Yes

A10: Yes
A11: Yes
A12: Yes
A13: Yes
A14: Yes
A15: Yes
A16: Yes
A17: No, because the time that a person does to help this study is quite a while.

Question 9: Do you have any additional comments?

A1: No
A2: No
A3: No
A4: No
A5: Would like to hear about the study outcome
A6: Glad to be involved
A7: The FFQ and Q39 no option for baking roasting which is how I often cook vegetables
A8: No
A9: No
A10: No
A11: Nil
A12: No
A13: No
A14: No
A15: No
A16: The only thing I found that it was not attending the university it was ongoing at home therefore I think participant should be offered more.
A17: No

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