Ageing, inorganic nitrate and vitamin C

Effects on markers of cardiovascular risk

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Abstract

The first stage of atherosclerosis is endothelial dysfunction (ED) which occurs at sites where the endothelial cell layer is exposed to injury or stress. Nitric oxide (NO) is an important signaling molecule secreted by the endothelium which regulates function of this vascular barrier. Reduced NO availability is a hallmark of ED. In contrast, inorganic nitrate and vitamin C may boost NO availability.

Data synthesis from 14 randomised controlled trials (RCTs) demonstrated that supplementation with inorganic nitrate or beetroot (a rich source of nitrate) improved flow mediated dilation (FMD) significantly. However, the improvement in FMD tended to be smaller in older, overweight/obese participants and in those with underlying cardio-metabolic disease. An umbrella review of 10 meta-analyses revealed weak evidence for an overall effect of vitamin C supplementation on biomarkers of cardiovascular disease (CVD) risk but subgroup and meta-regression analyses indicated significant benefits in participants with advanced age, higher BMI, lower vitamin C intake and higher CVD risk.

In the first crossover RCT, I tested the effects of a single dose of inorganic nitrate on markers of vascular function (skin microvascular blood flow and circulatory biomarkers) in younger and older obese individuals subjected to acute hyperglycaemia. Inorganic nitrate supplementation produced greater improvements in biomarkers of inflammation (interleukin-6) and oxidative stress (3-nitrotyrosine) in older compared with younger participants. Conversely, greater improvement in biomarkers of endothelial function (P- and E-selectin) was observed in younger rather than older participants.

In the second RCT, younger and older non-obese participants were supplemented with inorganic nitrate and vitamin C in a 2x2 factorial design. I found that the administration of inorganic nitrate and vitamin C, individually or combined, significantly improved blood pressure (BP), arterial stiffness and heart rate variability (HRV) indices in older participants. Moreover, inorganic nitrate significantly modified the effects of vitamin C on diastolic BP and HRV in younger participants. Finally, inorganic nitrate and vitamin C co-supplementation yielded synergistic effects on arterial stiffness, HRV indices and circulatory biomarkers.

In conclusion, age, obesity and metabolic disorders modify the cardiovascular effects of inorganic nitrate and vitamin C.

Supervisors' Certificate

This is to certify that the entitled thesis "Ageing, inorganic nitrate and vitamin C: Effects on markers of cardiovascular risk" has been prepared under my supervision at the Institute of Cellular Medicine/Newcastle University for the degree of PhD in Nutrition.

Signature

Supervisor: Prof John C. Mathers

Date:

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Supervisor: Dr Mario Siervo

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Student: Ammar Waham Ashor

Date:

Dedication

To my Family,

To my Country,

I dedicated this work

"We are like dwarfs sitting on the shoulders of giants. We see more, and things that are more distant, than they did, not because our sight is superior or because we are taller than they, but because they raise us up, and by their great stature add to ours."

John of Salisbury

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List of Abbreviations

3-NT	3-nitrotyrosine
8-oxodG	8-oxo-2-deoxyguanosine
ADMA	Asymmetric dimethyl L-arginine
AFR	Ascorbate free radicals
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AUC	Area under the curve
BH4	Tetrahydrobiopterin
BHF	British Heart Foundation
BMI	Body mass index
BP	Blood pressure
CARU	Clinical Ageing Research Unit
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart diseases
CI	Confidence interval
CIMT	Carotid intima media thickness
CRBs	Cerebrovascular diseases
CRP	C-reactive protein
CVD	Cardiovascular diseases
C-X-C motif	C reactive protein and chemokine
DASH	Dietary Approaches to Stop Hypertension
ED	Endothelial dysfunction
EDHFs	Endothelium derived hyperpolarizing factors
EF	Endothelial function
EFSA	European Food Safety Authority
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FBF	Forearm blood flow
FFQ	Food frequency questionnaire
FMD	Flow mediated dilatation
FMN	Flavin mononucleotide
GC-MS	Gas chromatography mass spectrometry
GLUT-4	Glucose transporter 4
GPx	Glutathione peroxidase
GSTs	Glutathione S-transferases
GxR	Goat anti-rabbit
Hr	Hour
HbA1c	Haemoglobin A1c
HDL	High density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HF	High frequency
HPLC	High performance liquid chromatography
HR	Hazard ratio
HRV	Heart rate variability
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
IL6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IVUS	Intravascular Ultrasound
Kcal	Kilo calorie
Kg	Kilogram
LDF	Laser Doppler flowmetry
LDI	Laser Doppler Iontophoresis
LDL	Low density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LF	Low frequency
MCP-1	Monocyte chemoattractant protein
METs	Metabolic equivalent of tasks
MJ	Millijoule
Mm	Millimetre
mmHg	Millimetre of mercury
MONICA study	Multinational Monitoring of Trends and Determinants in Cardiovascular Disease
MSD	Meso Scale Discovery

NADPH	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide-adenine-dinucleotide phosphate
NF-κB	Nuclear factor-κB
NHANES	National Health and Nutrition Examination Survey
nNOS	Neuronal nitric oxide synthase
NO_2^-	Nitrite
NO_3^-	Nitrate
NO	Nitric oxide
NOS	Nitric oxide synthase
NOx	Plasma nitrate/nitrite
OCT	Optic Coherence Tomography
OD	Odd ratio
OGTT	Oral glucose tolerance test
$ONOO^-$	Peroxynitrite
OQAQ	Overview of Quality Assessment Questionnaire
oxLDL	Oxidised low density lipoprotein
PAD	Peripheral arterial diseases
PAI-1	Plasminogen activator inhibitor
PBMCs	Peripheral blood mononuclear cells
PGI_2	Prostaglandin I_2
pNN50	Percentage of intervals that differ from each other by more than 50ms
pNpp	P-nitrophenyl phosphate
PPH	Post-prandial hyperglycaemia
PU	Perfusion unit
PWA	Pulse wave analysis
PWV	Pulse wave velocity
RCT	Randomised controlled trial
RMSSD	Square root of the mean of the squared differences of successive normal R-R intervals
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Relative risk
SD	Standard deviation
Sec	Second
SEM	Standard error of the mean
SMD	Standardised mean difference
SMR	Standardized mortality ratio
SOD	Superoxide dismutase
SVCT	Sodium-dependent vitamin C transporters
TG	Triglycerides
tPA	Tissue plasminogen activator
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VOP	Venous occlusion plethysmography
WHO	World Health Organization
WMD	Weighted mean difference

Structure of the thesis

The first chapter of this thesis introduces endothelial function (EF) and its relevance to the incidence and progression of cardiovascular diseases. I discuss the role of nitric oxide (NO) in maintaining vascular health and introduce the concept that oral inorganic nitrate and vitamin C supplementations may boost NO availability.

In the second chapter, I present a systematic review and meta-analysis of randomised controlled clinical trials which investigated the effects of inorganic nitrate and beetroot (a rich source of nitrate) supplementation on physiological measures of EF (flow mediated dilatation, forearm blood flow, skin microvascular blood flow, pulse wave velocity and augmentation index).

In the third chapter, I conducted an umbrella review of meta-analysis studies which have investigated the effects of vitamin C supplementation on biomarkers of cardiovascular disease risk (arterial stiffness, blood pressure, EF, glycaemic control and lipid profile).

In Chapter 4, I report the results of a randomised controlled crossover study which tested the effects of a single dose of inorganic nitrate on markers of vascular function (skin microvascular blood flow and circulatory biomarkers) in young and older obese individuals subjected to acute hyperglycaemia.

In the fifth chapter, I report the results from a second randomised controlled crossover study, also carried out in younger and older, but non-obese, participants. In this study, I investigated the effects of single doses of inorganic nitrate and vitamin C supplementations (alone or in combination) on blood pressure and vascular function (skin microvascular blood flow and pulse wave velocity).

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

Chapter 1. Introduction

1.1 Cardiovascular diseases

1.1.1 *Definitions*

Cardiovascular diseases (CVD) are characterised by a multifactorial pathogenesis. They can be divided according to the location of the main insult in the vascular bed. Insults affecting blood supply to the heart are referred to as coronary heart diseases (CHD). Pathologies affecting brain vessels are known as cerebrovascular diseases (CRB), whereas disorders of the peripheral arteries are termed as peripheral arterial diseases (PAD) (Frayn and Stanner, 2005).

The clinical outcomes of CHD, CRB, and PAD are largely attributed to a reduction in the blood supply to associated organs and tissues. This occurs secondary to thickening in the walls and functional obstruction of the arteries. The process of thickening is called atherosclerosis and the lesion formed is called plaque. The plaque may obstruct blood supply to the tissue partially causing ischemia (e.g. angina when it affects the heart muscle). Occasionally, the plaque may rupture and thrombosis may ensue leading to complete blockage of blood supply to, and death of, the tissue (e.g. myocardial infarction) (Gaziano, 2007).

1.1.2 *Epidemiology*

Cardiovascular diseases are the leading cause of death worldwide, accounting for around one third of all-cause mortality each year. Cardiovascular mortality varies between countries; relatively low mortality rates are observed in Japan and Mediterranean countries such as France, Spain and Portugal. Eastern European countries such as Ukraine, Russia and Latvia experience high CVD prevalence. Globally, around 45% of CVD deaths are due to CHD (WHO, 2013).

In the UK, cardiovascular mortality is amongst the highest in the world. It accounts for 19 and 28% of premature deaths among women and men, respectively. CHD is the most common cause of death in the UK with one in five men and one in ten women dying from CHD each year. Furthermore, CVD is also a major cause of morbidity and disability. It has been estimated that 1.5 million of the UK population have survived heart attack and 2.6 million have CHD (BHF, 2012). However, recently, the death rates from CHD have been falling across all age groups with faster rates observed in those aged over 55 years (43% reduction in men aged 55-64 in comparison with only 21% reduction observed in those aged 35-44 years) (BHF, 2012).

1.1.3 *Risk Factors for CVD*

The risk factors for CVD may be divided into modifiable and non-modifiable risk factors. The non-modifiable risk factors include age, sex and family history of cardiovascular illnesses (Mosca *et al.*, 2004). On the other hand, the modifiable risk factors are the major contributors to cardiovascular morbidity and mortality and account for > 90% of all myocardial infarctions (Dahlof, 2010). Importantly, manipulation of these modifiable factors changes CVD incidence (Ritz, 2007) (see Table 1.1).

Table 1.1: Risk factors for Coronary heart diseases (George and Lyon, 2010)

Modifiable factors		**Non-modifiable factors**
Lifestyle	Biochemical and physiological	Personal factors
• 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 (BP) • Diabetes mellitus • Thrombotic factors	• Age • Gender • Family history • Genetics

1.2 Atherosclerosis

1.2.1 *Definition*

Atherosclerosis is a multifactorial, progressive lesion of the arterial wall characterised by a soft atheros (lipid core) and surrounded by hard, collagenous fibrous tissue (sclerosis) (Channon, 2006). The atherosclerotic process may commence silently in late adolescence with the clinical manifestation usually becoming obvious after the age of 45 years (Wilson, 2004).

1.2.2 *Pathogenesis*

Atherosclerosis affects primarily the inner layer (intima) of blood vessels. It can affect arteries of different sizes from the aorta down to the small tertiary branches of the coronary arteries (Hansson, 2005). Historically, there were two major theories to explain the mechanism of atherosclerosis. The cellular theory proposes that the atherosclerotic lesion occurs secondarily to changes within the arterial wall. This theory called the response-to-injury hypothesis was proposed first by Rudolf Virchow in 1856 (Cullen *et al.*, 2005). The second theory is the humoral theory which stresses that changes in the environment surrounding the artery cause the

developing atherosclerotic plaque (Cullen *et al.*, 2005). Currently, atherosclerosis is described as a chronic inflammatory process of the arterial wall that occurs at sites exposed to continuous trauma due to turbulent blood flow. Consequently, both cellular and humoral factors contribute to the progression of atherosclerotic lesions (Stylianou *et al.*, 2012).

1.2.3 ***Stages of Atherosclerosis***

The first stage of atherosclerosis is endothelial dysfunction (ED) which occurs at sites where the endothelial cell layer is exposed to injury or stress. It has been suggested that reduction of nitric oxide (NO) availability is the hallmark of ED (Yetik-Anacak and Catravas, 2006). This is followed by structural alterations, including disruption of the luminal layer with the exposure of the underlying proteoglycan. The exposed proteoglycan permits sub-endothelial accumulation of LDL and its subsequent oxidative modifications by free radicals in the vascular wall (Weber and Noels, 2011).

The development of a fatty streak is the next stage in the process of atherosclerosis. This commences when the oxidised LDL triggers an inflammatory reaction in the endothelium which secretes cytokines in response. These cytokines will further attract T cells and leukocytes to the site of injury. The fatty streak lesion occurs when monocytes and macrophages engulf oxidised LDL forming lipid-laden foam cells (Rosenfeld, 2013). Lastly, successive accumulation of macrophage foam cells and extracellular lipid droplets form a core region surrounded by fibrous tissue composed of collagen and smooth muscle cells. This is called a fibroatheromatous plaque (Hansson *et al.*, 2006).

1.2.4 ***Risk Factors***

The coexistence of one or more of the risk factors (hyperlipidaemia, hyperglycaemias, obesity, hypertension, smoking, or family history of cardio-metabolic disorders) with local haemodynamic factors could contribute to initiation and progression of atherosclerosis (Figure 1.1) (Rodrigues *et al.*, 2012).

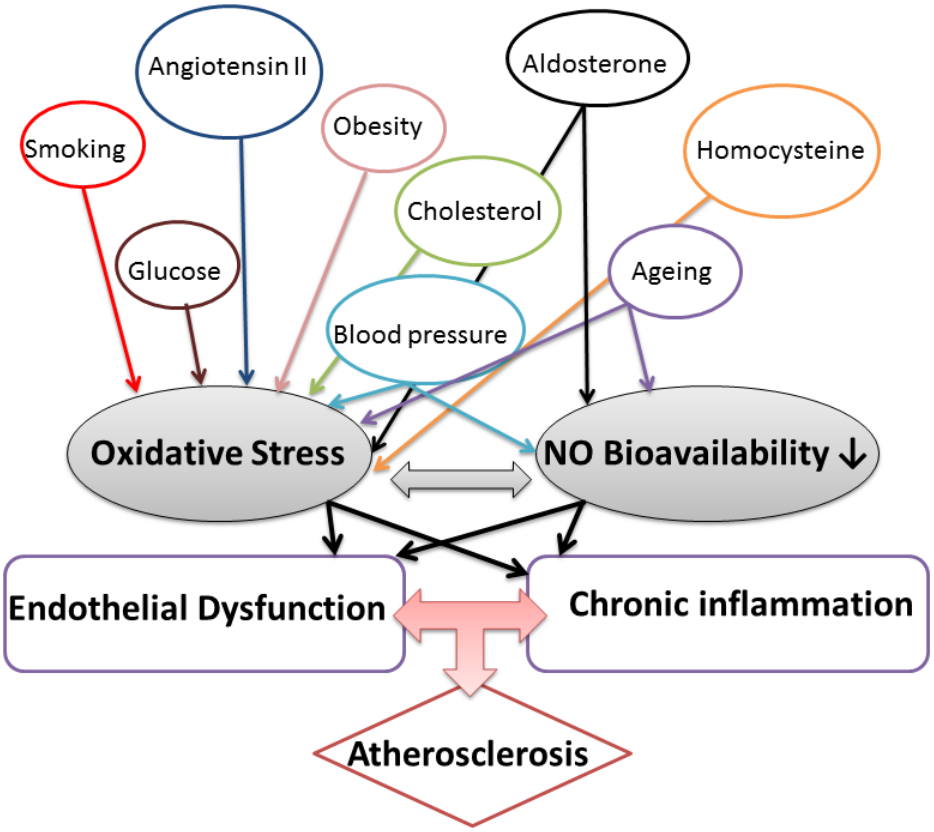


Figure 1.1: Risk factors for the development of atherosclerosis

1.2.5 *Clinical assessment of atherosclerosis*

The clinical assessment of atherosclerosis and differentiation of the various stages is important to evaluate individual risk for CVDs as well as for monitoring the effectiveness of clinical treatments. Various methods are used which differ in characteristics including equipment, cost, expertise, invasiveness, technical assumptions, rapidity, accuracy and precision. The most commonly applied methods are: 1) Carotid Intima Media Thickness (CIMT): a widely used and well-known marker of systemic atherosclerosis. A previous meta-analysis demonstrated that baseline CIMT predicts future CVD risk (Lorenz *et al.*, 2007); 2) Pulse Wave Analysis (PWA): commonly measured at the right radial artery. This approach measures the speed of conduction of heart waves across the arterial tree, the shorter the time of travel, the stiffer the arteries due to atherosclerosis (Holewijn *et al.*, 2010); 3) Intravascular Ultrasound (IVUS): This involves invasive catheter-based technology that allows the visualisation of all three layers of arterial wall (Klingenberg *et al.*, 2012); 4) Optical Coherence Tomography (OCT): This is a novel technology which uses a catheter-based light-source with a better resolution and lower penetration than IVUS (Klingenberg *et al.*, 2012).

1.3 The Endothelium

1.3.1 *Definition*

The endothelium is a monolayer of cells separating the vascular lumen from the rest of the blood vessel. It can be represented as one of the major organs of the human body with a surface area extending over 700 m^2 (Lehr *et al.*, 2006). The endothelium had been regarded as an inert physical barrier between the circulating blood and the underlying tissue. It is now recognised that the endothelium has vital paracrine, endocrine and autocrine functions (Sena *et al.*, 2013). Therefore, in addition to helping maintain blood flow, the main function of the endothelium is to serve as an endocrine organ (Michiels, 2003).

1.3.2 *Functions of the endothelium*

The endothelium generates several extracellular messengers that mediate multiple functions including preserving haemostatic balance. In addition to insulating the thrombogenic sub-endothelial layers, the endothelium secretes factors that inhibit the inappropriate formation of thrombus, e.g. NO, prostacyclin I_2 (PGI_2), tissue plasminogen activator (tPA) and protein C/protein S. However, in case of vessel damage and exposure to certain pro-inflammatory substances, the balance is shifted towards a procoagulant/prothrombotic state. This stimulates the endothelium to secrete agents that help with platelet aggregation and clot formation including platelet activating factor, von Willebrand factor, and thromboxane A_2 (Sumpio *et al.*, 2002). Furthermore, NO secreted by the normal endothelium prevents an inflammatory response in the vascular wall following local injury. In contrast, a dysfunctional endothelium secretes adhesion molecules and chemokines that attract inflammatory cells to the site of injury (van Hinsbergh, 2012).

Normal vascular endothelium has anti-proliferative and anti-apoptotic properties that are mediated through the activity of NO, PGI_2 and C-type natriuretic peptide. Moreover, endothelial cells secrete factors that promote proliferation of smooth muscle cells and the formation of new blood vessels, e.g. vascular endothelial growth factor (VEGF), angiopoietins and adropins (Mensah, 2007). Dysfunction in the endothelium is characterized by: disturbed vasodilator and anticoagulant function, increased adhesiveness of the vessel wall for platelets and leukocytes (inflammation), reduced fibrinolytic activity and breakdown of barrier function causing leakage and oedema formation (Vanhoutte *et al.*, 2009).

1.4 Factors affecting endothelial function (EF)

1.4.1 *Nitric oxide*

NO is a free radical gas molecule which is involved in many physiological processes and reduced availability of NO contributes to many pathological conditions (Yetik-Anacak and Catravas, 2006). NO is regarded as one of the most important molecules secreted by the endothelium. It is a highly diffusible molecule with a very short half-life (<1 sec) (Channon, 2006). The production of NO is catalysed by the nitric oxide synthase enzyme (NOS). There are three isoforms of this enzyme, endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Lei *et al.*, 2013). eNOS is a homodimeric enzyme expressed constitutively in endothelial cells where it facilitates the conversion of the amino acid L-arginine into L-citrulline and NO (Michiels, 2003). This process requires molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates, as well as the following cofactors: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH4), heme and calcium-calmodulin (Figure 1.2) (Forstermann and Sessa, 2012).

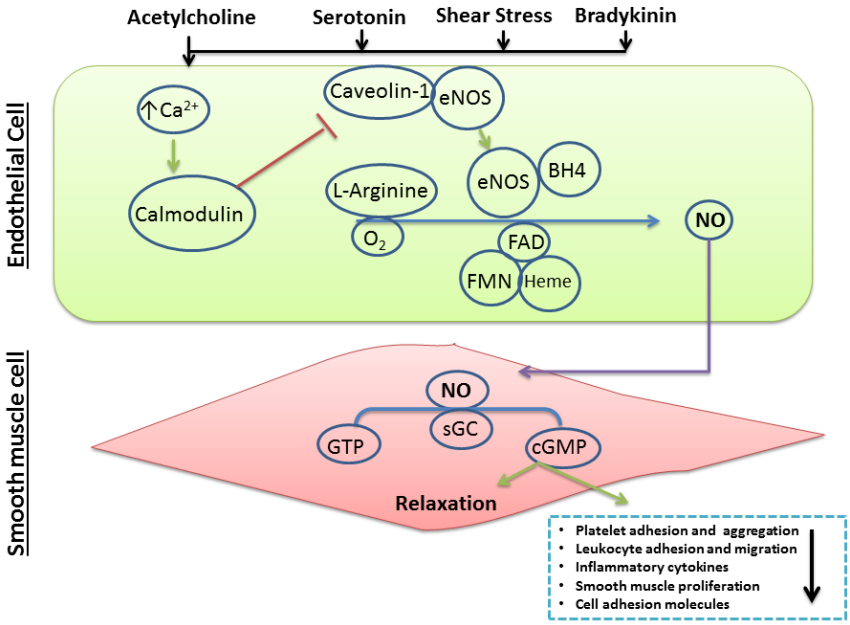


Figure 1.2: Mechanisms of activation of endothelial nitric oxide synthase (eNOS) and the release of nitric oxide (NO). FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; BH4, tetrahydrobiopterin; sGC, soluble guanylyl cyclase

The trigger for NO synthesis and release is either mechanical stretching of the vessel wall or receptor-mediated agonists such as bradykinin, acetylcholine or histamine (Khazaei *et al.*,

2008). These signals lead to an increase in intracellular calcium concentration. Intracellular Ca^{2+} binds to calmodulin to form Ca^{2+}-calmodulin complex that mobilises eNOS from its binding to Caveolin, so allowing the activated (eNOS) to catalyse synthesis of NO from L-arginine (Khazaei *et al.*, 2008). Because of the gaseous nature of NO, it diffuses from where it is synthesised in the endothelium to the vascular smooth muscle where it activates soluble guanylate cyclase leading to increasing intracellular cyclic guanosine monophosphate (cGMP). The cGMP causes smooth muscle relaxation and, eventually, arterial dilation (Forstermann, 2010).

In addition to arterial dilation, NO has many other vital protective functions in blood vessels. These protective functions include decreasing: 1) smooth muscle proliferation; 2) platelet aggregation; 3) endothelin production; 4) monocyte and platelet adhesion; 5) expression of adhesion molecules and 6) oxidation of LDL (Vanhoutte *et al.*, 2009). Because of the vital role of NO, researchers have suggested that reduced NO availability is the major cause of ED. This deficiency activates atherogenic processes in the vessel wall which include vasoconstriction, monocyte activation and adherence to vascular endothelium, proliferation of smooth muscle cells, thrombosis and impaired coagulation and, eventually, atherosclerosis (Versari *et al.*, 2009).

Many factors modulate NO synthesis and degradation and, therefore, affect EF. Asymmetric dimethyl L-arginine (ADMA), is a product of protein metabolism formed secondarily to methylation of L-arginine (Giles, 2006). ADMA decreases the synthesis of NO by reducing the expression and/or activity of eNOS. ADMA is increased in many pathological conditions such as hypercholesterolaemia, atherosclerosis, hypertension, chronic heart failure, diabetes mellitus and chronic renal failure (North and Sinclair, 2012). Furthermore, uncoupling of eNOS as a result of the oxidation of BH4 or depletion of L-arginine and the accumulation of endogenous methylarginines may lead to reduced formation of NO, i.e. the eNOS enzyme is converted from a NO-producing enzyme to a superoxide-producing enzyme (Schmidt and Alp, 2007). Overproduction of reactive oxygen species (ROS) is the major cause of reduced NO availability in CVD. NO reacts with the superoxide anion with high affinity forming the harmful free radical peroxynitrite ($ONOO^-$) (Sena *et al.*, 2013). Lipid peroxyl radicals and oxidised LDL react with endothelial NO before it reaches the vascular smooth muscle cells and, therefore, inhibit NO from dilating blood vessels (Sena *et al.*, 2013).

1.4.2 *Oxidative stress*

Oxidative stress is the condition of excessive production of ROS beyond the neutralising capacity of the endogenous antioxidant defence mechanism. ROS, or free radicals, are atoms or group of atoms with one or more electrons missing. The missing electrons make these molecules highly reactive as they try to capture electron from other compounds to gain stability. Raised concentrations of free radicals have detrimental effect on cellular function because ROS interact with and damage membranes, enzymes and nucleic acids (Packer, 2005).

Sources of ROS in CVD include the following: 1) mitochondrial respiratory chain: the mitochondria are a well-known source of ROS. About 1% of the O_2 consumed by the mitochondrion is reduced by single electrons to form the free radical superoxide O_2^- (Murphy, 2009); 2) NADPH oxidases: these are a family of multiple-subunit enzymes located in the membranes of various cells e.g. endothelial cells, smooth muscle cells and fibroblasts. NADPH oxidases generate superoxide via one electron reduction of oxygen using NADPH as an electron source. These enzymes are upregulated in disorders including hypertension, diabetes and hypercholesterolaemia (Munzel *et al.*, 2010); 3) xanthine oxidase: this enzyme is highly expressed in capillary endothelium and readily donates electrons to molecular oxygen thereby generating the superoxide free radical. Additionally, the xanthine oxidase can reduce nitrate (NO_3^-) to nitrite (NO_2^-) to facilitate the production of nitric oxide (Munzel *et al.*, 2010); 4) lipoxygenases: these enzymes catalyse the incorporation of oxygen into polyunsaturated fatty acids. Some lipoxygenase isoforms promote atherosclerosis by generating ROS and causing oxidative modification of LDL (Sugamura and Keaney, 2011); 5) nitric oxide synthases: eNOS may become uncoupled which leads to a reduction of molecular oxygen rather than a transfer of electrons to L-arginine, therefore generating superoxide instead of NO (Sugamura and Keaney, 2011).

On the other hand, the antioxidant mechanisms of the human body include: 1) superoxide dismutase (SOD): SOD catalyses the dismutation of O_2^- into oxygen and hydrogen peroxide. A variant of this enzyme (SOD3) is associated with high risk of CHD (Forstermann, 2010); 2) catalase: this enzyme catalyses the decomposition of hydrogen peroxide to water and oxygen (Forstermann, 2010); 3) glutathione peroxidase (GPx): GPx reduces free hydrogen peroxide to water and lipid hydropreoxides to their corresponding alcohols (Forstermann, 2010); 4) heme oxygenase: the cardiovascular protective effect of this enzyme is through its ability to degrade the pro-oxidative heme to biliverdin. Biliverdin is subsequently changed to bilirubin, which has antioxidant effects (Forstermann, 2010); 5) thioredoxin: thioredoxins are a class of small redox proteins, present in the endothelium and smooth muscle cells, which have the ability to

scavenge hydrogen peroxide and peroxynitrite (Sugamura and Keaney, 2011); 6) paraxonase: this family of enzymes, which are synthesised in the liver and circulate in plasma, have the ability to reduce the peroxidation of HDL and LDL (Sugamura and Keaney, 2011).

Several biomarkers in the blood can be used to estimate the degree of oxidative stress. In healthy individuals, the circulating concentration of oxidised low density lipoprotein (oxLDL) is an independent risk factor for CHD (Forstermann, 2010). Plasma F_2-isoprostanes are considered a valid measure of oxidative stress and increased urinary output of isoprostanes is regarded as an independent risk factor for CVD (Forstermann, 2010).

1.4.3 ***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 rest of the human body. For example, chronic smoking deteriorates EF by decreasing NO production and enhancing its degradation via the generation of oxygen free radicals (Toda and Toda, 2010). Furthermore, hypercholesterolemia and high homocysteinaemia may reduce the availability of NO secondary to oxidative stress (Dayal and Lentz, 2005; Sibal *et al.*, 2010).

ED has been demonstrated both in resistance and conduit arteries of several animal models of hypertension (Tang and Vanhoutte, 2010). Moreover, reduced forearm blood flow responses to endothelium-dependent vasodilator agonists, such as acetylcholine and bradykinin (Linder *et al.*, 1990; Panza *et al.*, 1990) and increased vasoconstrictor responses to locally administered NOS inhibitors (Panza *et al.*, 1993) were observed in hypertensive patients. In their prospective cohort study, Rossi *et al.* (2004) revealed that a 1 unit reduction in flow mediated dilatation (FMD) is associated with a 16% increase in the risk of hypertension in postmenopausal women, independent of age and baseline blood pressure. The cause of ED associated with hypertension is speculated to be reduction in NO bioavailability (increased degradation by oxidative stress, reduced production by eNOS inactivation) and abundance of vasoconstrictor agents in the circulation such as angiotensin II and prostaglandins (Tang and Vanhoutte, 2010).

1.4.4 ***Obesity, hyperglycaemia and ED***

The prevalence of obesity has dramatically increased worldwide and nowadays, obesity considered as a global epidemic (Ng *et al.*, 2014). Obesity is a major risk factor for numerous diseases and conditions including CVD, type 2 diabetes, hypertension, cancer and sleep apnoea (Bastien *et al.*, 2014). In the context of the present project, obesity is an independent risk factor

for atherosclerosis (Al Suwaidi *et al.*, 2001) and significant EF deterioration was observed in a cohort of severely obese children (Tounian *et al.*, 2001). Moreover, Arcaro *et al.* (1999) demonstrated that the visceral/subcutaneous adipose tissue ratio significantly predicts ED in obese individuals ($r = 0.624$, $P = 0.0058$). NO is reduced in obesity either because of enhanced degradation (oxidative stress) or decreased formation (eNOS inactivation) (Toda and Okamura, 2013).

Adipose tissue produces several molecules that influence EF including leptin, resistin, tumor necrosis factor alpha (TNF-α), interleukin (IL) 6, monocyte chemoattractant protein (MCP)-1, plasminogen activator inhibitor (PAI)-1, adiponectin and the proteins of the renin–angiotensin system (Avogaro and de Kreutzenberg, 2005).

Leptin affects EF by stimulating sympathetic activity (vasoconstriction), increasing oxidative stress and inflammatory markers. On the other hand, resistin increases the production of endothelin-1 and vascular adhesion molecules (Singhal, 2005). Moreover, the secretion of the other inflammatory mediators by the adipose tissue contributes to a chronic inflammatory state in the vascular wall and the subsequent ED (Avogaro and de Kreutzenberg, 2005).

Chronic hyperglycaemia (e.g. diabetes) is associated with greater CVD risk (Bakker *et al.*, 2009). Besides, evidence from epidemiological studies demonstrated that postprandial hyperglycaemia is a better predictor for cardiovascular mortality and morbidity than sustained hyperglycaemia (Mah and Bruno, 2012). Transient and prolonged hyperglycaemia impairs EF in animals (Bitar *et al.*, 2005) and humans (Ceriello *et al.*, 2002).

In a prospective population-based cohort (n=445, median follow-up of 7.6 years), reduced flow-mediated dilatation was associated with a higher rate of cardiovascular events in type 2 diabetes mellitus patients. The authors concluded that ED and type 2 diabetes mellitus act synergistically to increase cardiovascular event risk (van Sloten *et al.*, 2014). Hyperglycaemia is associated with increased oxidative stress, uncoupling of eNOS, LDL oxidation, and increased concentrations of vasoconstrictor mediators, e.g. endothelins, angiotensins, and prostanoids (Sena *et al.*, 2013).

1.5 Assessment of EF

Since ED precedes atherosclerosis and its degree of impairment reflects the severity of atherosclerosis (Harris *et al.*, 2010), the assessment of EF has important diagnostic, therapeutic

and prognostic value for atherosclerotic vascular diseases. The following methods can be used to assess EF:

1.5.1 *Invasive methods*

a. Intracoronary infusions of vasoactive agents: Infusion of vasoactive agents directly into the coronary arteries combined with quantitative angiography is the method of choice for direct quantification of coronary endothelial function (Higashi, 2015). It allows both the evaluation of dose-response relations of endothelial agonists and antagonists, as well as assessment of the basal EF by the infusion of NOS inhibitors (Tousoulis *et al.*, 2005). Although intracoronary studies are the gold standard for early detection of ED in the coronary arteries, they have the disadvantage of being invasive and expensive, with the risks of coronary catheterisation, and cannot be used as a screening test in the general population (Tousoulis *et al.*, 2005).

b. Intrabrachial infusion of vasoactive agents: Endothelial function in the coronary arteries is closely related to endothelial function in peripheral arteries such as the brachial artery (Tousoulis *et al.*, 1997). Therefore, to avoid intracoronary invasiveness, vasoactive substances can be injected into the brachial arteries to assess EF and the resulting measure of brachial artery EF reflects the health status of the coronary arteries (Tousoulis *et al.*, 2005).

1.5.2 *Non-invasive methods*

a. FMD: Many blood vessels respond to increased blood flow (shear stress) by NO-dependent dilation of that vessel. Estimating the degree of dilatation of the vessel by Ultrasound is called FMD (Corretti *et al.*, 2002). The principle of FMD is to occlude the forearm blood flow for 5 minutes with blood pressure cuff inflated to supra-systolic levels. Subsequent release of the cuff leads to increased shear stress stimuli, NO production and vasodilation. Assessment of the degree of vasodilation with high resolution ultrasound is the FMD (Charakida *et al.*, 2010). Advantages of FMD are that it is a safe, non-invasive, fast method and results for FMD relate closely to EF in the coronary arteries, however FMD measurement varies between assessors i.e. it is observer-dependent (Tousoulis *et al.*, 2005).

b. Venous occlusion plethysmography (VOP): Forearm blood flow (FBF) can be measured with VOP. The principle of this method is that occlusion of venous blood flow with a pressure cuff placed on the forearm leads to continuous flow of the arterial blood and linear increase in forearm volume over time (Wilkinson and Webb, 2001). The changes in FBF

during acetylcholine or nitroprusside infusions represent indices of endothelium dependent and endothelium independent dilation, respectively (Tousoulis *et al.*, 2005). A disadvantage of VOP is its semi-invasiveness (requires intra-arterial cannulation) but this technique is less observer-dependent than FMD (Tousoulis *et al.*, 2005).

c. Cutaneous microvascular blood flow: One of the major drawbacks of VOP and FMD is the assessment of total limb flow without distinguishing between the muscle and skin circulation, which have different functions (Newton *et al.*, 2001). The microcirculation refers to arteries, arterioles, capillaries, and venules with the smallest resistance and less than 150 μm in diameter (Roustit and Cracowski, 2013). Skin microcirculation is important because it is the place where fine control of the blood supply takes place; tissue ischemia is highly dependent on microvascular flow in most circumstances (Newton *et al.*, 2001). Patients with impaired coronary microvascular function also have evidence of impaired peripheral microvascular function, suggesting a generalized disorder in the regulation of the microvasculature (Roustit and Cracowski, 2012). Similarly, correlated abnormalities between cutaneous and retinal microcirculation in diabetic patients have been reported (Roustit and Cracowski, 2012).

Recently, the skin microcirculation has emerged as an accessible and potentially representative vascular bed to examine the mechanisms of microcirculatory function and dysfunction (Holowatz *et al.*, 2008). Minimally invasive skin-specific methodologies make the skin microcirculation a useful translational model for investigating mechanisms of vascular disease and providing preclinical data about the state of microcirculatory function in high-risk populations (Roustit and Cracowski, 2012). To date, the skin has been used as a circulation model to investigate vascular mechanisms in a variety of disease states, including hypertension, Type II diabetes, hypercholesterolemia, renal disease, PAD, atherosclerotic CAD, heart failure and ageing (Holowatz *et al.*, 2008).

Laser Doppler flowmetry measures skin microvascular EF. Laser Doppler is based on the reflection of a beam of laser light. Light undergoes changes in wavelength (Doppler shift) when it hits moving blood cells. The magnitude and frequency distribution of these changes in wavelength are related to the number and velocity of blood cells. Several different signals can be recorded but the red blood cell flux (i.e. the product of the velocity and concentration of moving blood cells within the measured volume) is used most often (Roustit and Cracowski, 2013).

NO secretion and its accompanied vasodilatation in the microcirculation can be enhanced either with the application of post-reactive hyperaemia in the forearm or through the introduction of acetylcholine through the skin (iontophoresis) (Roustit and Cracowski, 2013). Iontophoresis delivers charged pharmacological agents in a vehicle solution to a localized area of skin using opposing electrical current (Holowatz *et al.*, 2008). There are significant correlations between Laser Doppler flowmetry and plasma NO and endothelin-1 (Turner *et al.*, 2008). Additionally, a preliminary study showed a correlation between skin and coronary microvascular function (Turner *et al.*, 2008). The major advantage of this technique is its sensitivity at detecting and quantifying relative changes in skin blood flow in response to a given stimulus (Cracowski *et al.*, 2006). A disadvantage of Laser Doppler is the contribution of prostaglandins, in addition to NO, to the vasodilator response (Turner *et al.*, 2008). Reproducibility is a major issue of Laser Doppler flowmetry but this variation can be minimised if the recording site is standardised (Cracowski *et al.*, 2006).

d. Pulse wave analysis (PWA) and velocity (PWV): contraction of the left ventricle during systole generates a pressure wave that travels across the aortic wall and the highly conductive arteries. This pressure wave confronts impedance at the junction of the large conduit artery and high-resistance arterioles, blocking its entry into the arterioles so that it is reflected backwards towards the heart (Safar *et al.*, 2011). The contribution of the reflected wave to the aortic pressure can be estimated by calculating the augmentation index (AIx). The speed of travel of the heart wave between two arterial points is called pulse wave velocity (e.g. carotid-femoral PWV) and can be detected by using pressure transducers (Shirwany and Zou, 2010). PWV reflects structural changes in the vessel walls (arterial stiffness) and such structural and functional changes in the vessel wall contribute to the onset and progression of arterial stiffness (Zieman *et al.*, 2005).

Structural changes include the replacement of elastin with collagen and smooth muscle proliferation (Correia and Haynes, 2007). Oxidative stress and inflammation contribute significantly to this structural remodelling (Patel *et al.*, 2011) through the process of smooth muscle proliferation and collagen deposition (Park and Lakatta, 2012) which increase the rigidity of vascular conduits and modifies arterial compliance. Functional deterioration involves reduced NO bioavailability and the onset of endothelial dysfunction. Arterial stiffening is a hallmark of ageing and is closely associated with many pathological conditions including atherosclerosis, dyslipidaemia, diabetes and chronic kidney diseases (Shirwany and Zou, 2010). Arterial stiffness is a precursor of CVD and is a marker for increased CVD risk and all-cause mortality (Ben-Shlomo *et al.*, 2014).

1.5.3 ***Biochemical markers***

Circulating markers of EF which are secreted from the endothelium in response to inflammation or injury include E- and P-selectin, vascular cell adhesion molecules, and intercellular adhesion molecules (Deanfield *et al.*, 2007). Additionally, many direct and indirect methods have been used to assess the production of NO. *In vivo* NO production may reflect the underlying EF in health and disease (Siervo *et al.*, 2011b). Blood biomarkers can provide important information regarding mechanisms and severity of endothelial dysfunction in the population (Higashi, 2015). However, due to the biological variability, difficulty, expense and limited assay availability, these biomarkers currently have only a very limited role in the assessment of individual patients (Deanfield *et al.*, 2007).

Circulating levels of NO_2^- and nitrosylated proteins partially reflect endothelial generation of NO, but are difficult to measure and may not always represent endothelial NO production. Measurements of NO_2^- and 3-nitrotyrosine (3-NT) may be confounded by the formation of adducts from other nitrogen-containing species, other sources of NO, and wide variation in dietary NO (Deanfield *et al.*, 2007). 3-NT is the result of a post-translational modification in proteins carried by reactive nitrogen species such as peroxynitrite and nitrogen dioxide radicals (Teixeira *et al.*, 2016). It is formed after the substitution of a hydrogen by a nitro group (NO_2^-) in the ortho position of the phenolic ring of the tyrosine residues (Teixeira *et al.*, 2016). Moreover, tyrosine can be nitrated by peroxidase (or haemoprotein)-catalysed, hydrogen peroxide-dependent oxidation of nitrite to form NO_2^- (Bryan and Grisham, 2007).

Activated endothelial cells secrete a cascade of inflammatory cytokines (including E-selectin, vascular cell adhesion molecule 1, intercellular adhesion molecule 1 and P-selectin) that trigger leukocyte attraction, adhesion and migration into the sub-endothelial space. These molecules are fundamental to the initiation, progression and destabilization of the atherosclerotic lesion and can be measured in the circulation with commercial immunoassays (Constans and Conri, 2006). E-selectin is probably the most specific for endothelial cell activation. Studies showed that E-selectin increases in association with cardiovascular risk factors and such increases are associated with structural and functional measures of atherosclerotic disease, as well as with adverse cardiovascular prognosis (Deanfield *et al.*, 2007).

1.6 Nutritional interventions in cardiovascular diseases

Dietary habits are strongly associated with CVD incidence and progression (Verschuren, 2012). It has been speculated that most of the risk factors of atherosclerosis are influenced by diet (De

Caterina *et al.*, 2006; Rosenfeld, 2013) and, for example, there is considerable evidence for adverse effects of nutrients such as saturated and trans-fatty acids on EF (Hennig and Toborek, 2001).

1.7 Dietary modulation of EF

1.7.1 *Dietary patterns*

a. Positive energy balance and obesity are strongly associated with insulin resistance and chronic inflammation (secretion of pro-inflammatory cytokines). Insulin resistance and inflammatory cytokines are pro-atherogenic through mechanisms linked to ED (Hamdy *et al.*, 2003). Six weeks exposure to a very-low-calorie diet (daily energy: 580 kcal/2.3 MJ) improved FMD by 60% (Raitakari *et al.*, 2004). Moreover, 6-years caloric restriction in healthy individuals reduced CIMT by 40% in comparison with a control group (Fontana *et al.*, 2004).

b. The Mediterranean diet is characterised by high content of monounsaturated fats, fruits and vegetables, legumes, high-fibre cereals and antioxidants and low intakes of animal-derived foods apart from fish. Feeding the Mediterranean diet for 2 months to obese people was found to significantly improve EF (2.05%; 95% CI: 0.97, 3.13%) (Rallidis *et al.*, 2009). Furthermore, the Mediterranean diet was found to be anti-atherogenic and significantly reduce the incidence of cerebro- and cardiovascular diseases (Martínez-González *et al.*, 2011; Estruch *et al.*, 2013). The Mediterranean dietary intervention halts atherosclerosis through modulating the pro-inflammatory processes that initiate and disseminate ED, plaque formation and rupture (De Caterina *et al.*, 2006).

c. The Dietary Approaches to Stop Hypertension (DASH) diet is a widely-used life style intervention to reduce CVD risk. A recently published study showed that a four week DASH diet-intervention enhanced EF by 68% in middle-aged hypertensive patients (Jablonski *et al.*, 2013). The proposed mechanism is reduction of oxidative stress and enhancement of eNOS expression (Jablonski *et al.*, 2013).

1.7.2 *Macronutrients*

a. Dietary fats: Eating meals rich with saturated fat significantly deteriorates EF in healthy volunteers (FMD: -2.2 ± 0.9%; $p < 0.05$). Moreover, high-fat meals deteriorate the anti-inflammatory properties of HDL in terms of their ability to inhibit expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Aromataris *et al.*, 2015). Epidemiological studies revealed an inverse

relationship between the dietary consumption of fish and mortality from coronary heart diseases (Kris-Etherton *et al.*, 2002). Moreover, a recently published meta-analysis demonstrated beneficial effects of fish oil on EF (FMD: 1.49%; 95% CI 0.48% to 2.50%) (Xin *et al.*, 2012) and the authors speculate that these beneficial effects were due to the antioxidant and anti-inflammatory properties of the fish oils (Xin *et al.*, 2012).

b. Carbohydrates: In contrast with high-fat, low-carbohydrate, acute ingestion of a low-fat, high carbohydrate meal did not deteriorate EF significantly (Ong *et al.*, 1999). However a recently published meta-analysis revealed that meal ingestion (irrespective of carbohydrate content) significantly reduces FMD (-2.03; 95% CI: -2.28, -1.77) (Thom *et al.*, 2016).

c. Proteins: Studies in mice have shown that a high-protein intake is associated with endothelial dysfunction and progression of atherosclerosis (Foo *et al.*, 2009; Namikoshi *et al.*, 2009). Moreover, 1 year follow-up of 26 patients on high-protein diet showed significant deterioration in coronary blood flow and circulatory inflammatory biomarkers (Fleming, 2000).

1.7.3 ***Micronutrients***

a. Observational studies have provided strong evidence of an inverse correlation between dietary intake of antioxidant vitamins and risk of cardiovascular morbidity and mortality (Rimm *et al.*, 1993; Yochum *et al.*, 2000). However, randomised controlled trials (RCTs) testing the effects of antioxidant supplements on similar outcomes has not reported significant protective effects. In the study by (Sesso *et al.*, 2008), neither vitamin C nor vitamin E supplementation for 8 years to male physicians reduced the risk of major cardiovascular events. Lack of beneficial effects was also observed in post-menopausal women given antioxidant vitamins (Waters *et al.*, 2002). Further evidence regarding the effects of vitamin C on cardiovascular risk biomarkers will be discussed in Chapter three of this thesis.

b. Because the semi-essential amino acid L-arginine is of vital importance for endogenous NO synthesis, there has been speculation that it has important protective effects on EF (Gornik and Creager, 2004). Data synthesis of 11 RCTs (387 participants) demonstrated that compared with placebo, L-arginine intervention significantly lowered systolic BP by 5.39 mmHg (95% CI −8.54 to −2.25, $P = .001$) and diastolic BP by 2.66 mmHg (95% CI −3.77 to −1.54, $P < .001$) (Dong *et al.*, 2011). Another meta-analysis (12 studies, 492 participants) revealed that L-arginine supplementation significantly enhanced EF (FMD: 1.98%; 95% CI: 0.47, 3.48) (Bai *et al.*, 2009).

c. Omega-3 fatty acids may improve EF by reducing inflammatory cytokines and enhancing the release of NO. Recently, a meta-analysis study (16 eligible studies involving 901 participants) showed that omega-3 fatty acids, at doses ranging from 0.45 to 4.5 g/d over a median of 56 days, significantly improved EF (FMD: 2.30%; 95% CI: 0.89–3.72%) (Wang *et al.*, 2012).

d. Data from several studies have shown that eating diets rich in polyphenols is associated with better cardiovascular prognosis (Stoclet *et al.*, 2004). The observed improvement was suggested to be secondary to improvement in EF. Polyphenols may enhance EF by several mechanisms, e.g. reducing oxidative stress and enhancing NO production (Schini-Kerth *et al.*, 2010). Dietary flavonoids (which are examples of polyphenolic compounds) that occur naturally in plant foods increased FMD after acute (3.99%; 95% CI: 2.86, 5.12; 6 studies) and chronic (1.45%; 0.62, 2.28; 2 studies) intake (Hooper *et al.*, 2008).

e. Poor vitamin D status is associated increased risk of cardiovascular events and mortality (Pludowski *et al.*, 2013). Furthermore, both observational and interventional studies have established an association of low vitamin D concentration with ED (Caprio *et al.*, 2012). However, pooling data from 16 clinical trials showed no significant effect of vitamin D on EF overall (standardised mean difference [SMD]: 0.08, 95% CI: -0.06, 0.22, P=0.28). However, subgroup analysis showed a significant improvement of EF in diabetic subjects (SMD: 0.31, 95% CI: 0.05, 0.57, P=0.02). Meta-regression analyses demonstrated that the effect size was weakly associated with diastolic BP (β: 0.02; P=0.07) and body mass index (BMI) (β: 0.05; P=0.06) (Hussin AM *et al.*, 2015).

f. Folic acid and B vitamins may enhance EF through their capacity to reduce homocysteine concentrations (Chambers *et al.*, 2000). A meta-analysis of RCTs showed that folic acid enhanced EF significantly (FMD:1.08; 95% CI: 0.57,1.59) (de Bree *et al.*, 2007).

1.8 Vascular ageing

The following sections (1.8, 1.9 and 1.10) are adopted from our book chapter "Chapter 43 - Vitamin C, Antioxidant Status, and Cardiovascular Aging, in Mocchegiani, E. (ed.) Molecular Basis of Nutrition and Aging. San Diego: Academic Press, pp. 609-619"

1.8.1 *Overview of the biology of ageing*

The ageing process is characterised by a progressive decline of cellular integrity and function resulting from the structural modification of macromolecules including formation of oxidised lipid species, advanced glycated products, nitrosylated proteins and DNA mutations and

epimutations. Epimutation is any heritable change such as methylation that did not affect the actual sequence of the DNA (Dobrovic and Kristensen, 2009; Vijg and Suh, 2013). The accumulation of modified molecules and their incorporation into cellular components are responsible for the structural and functional deterioration of tissues and organs with time (Ashor *et al.*, 2016b).

Whilst the complexity of the biological mechanisms contributing to the ageing process is still poorly understood, a comprehensive summary of some of these mechanisms has been proposed recently by Lopez-Otin *et al.* (2013). These authors proposed the following set of hallmarks of ageing viz. genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication which are seen across diverse species (Lopez-Otin *et al.*, 2013). Many factors contribute to age-related molecular damage but it seems likely that much damage is due to three common stressors viz. oxidative stress/ redox changes, inflammation and metabolic stress (Mathers, 2015).

The accumulation of molecular damage in stem cells which leads to cell death, senescence or loss of regenerative function is likely to have a particularly profound effect on ageing because of the role of stem cells in maintaining tissue integrity. The cumulative load of (macro) molecular damage, together with alterations in tissue cellularity, causes diminished functional capacity and increases the risk of age-related frailty, disability and disease, including CVD. In humans, factors such as nutrition which modulate the ageing trajectory and the risk of age-related diseases must do so by affecting the acquisition of molecular damage with age or by altering the body's ability to defend itself against that damage (Mathers, 2015). For example, nutrition influences DNA repair - a key cellular defence mechanism which ensures the integrity of the genome (Tyson and Mathers, 2007). Although the mechanisms through which dietary factors affect DNA repair systems are poorly understood, recent studies from the Mathers laboratory suggest that this may include altered expression of the genes encoding key repair proteins such as the base excision repair (BER) enzyme apurinic/apyrimidinic endonuclease 1 (APE1) and that this may involve epigenetic mechanisms (Langie *et al.*, 2014).

1.8.2 *Ageing and cardiovascular disease risk*

Cardiovascular diseases are the most common cause of death among elderly populations in the Western World (Virdis *et al.*, 2010). Age-specific mortality rates from heart disease and stroke increase exponentially with age and account for more than 40% of all deaths among individuals

aged 65-74 years and almost 60% at age 85 years and older (Ungvari *et al.*, 2010). In the UK, although death rates from CVD have been falling for 4 decades, ischemic heart disease is ranked number one for years of life lost due to premature mortality. Importantly, lifestyle factors including smoking, poor diet and lack of physical activity are the major causes of morbidity measured by disability-adjusted life years (Murray *et al.*, 2013).

As noted above, ageing is associated with complex structural and functional changes in all tissues including the vascular system and these changes increase CVD risk independent of other risk factors such as hypertension, diabetes or hypercholesterolemia (Jani and Rajkumar, 2006). These functional changes include wide-spread ED, dilation of the central arteries and increased arterial stiffness (Taddei *et al.*, 2001; Mitchell *et al.*, 2004). Development of strategies to attenuate ageing of the vascular system could make a substantial contribution to lowering CVD risk and improving the quality of life of older people (El Assar *et al.*, 2012).

1.8.3 ***Factors that impair EF with aging***

a. Oxidative stress

The accumulation of endogenous oxygen radicals and the consequent oxidative modification of cellular macromolecules (lipids, proteins and nucleic acids) have been suggested to contribute to ageing in all organisms (Puca *et al.*, 2013). Increased production of free radicals, secondary to mitochondrial dysfunction, causes oxidative damage to cells including vascular cells (Fusco *et al.*, 2007). As discussed earlier (Section 1.4.2), the accumulation of free radicals eventually causes reduced NO bioavailability and ED (Figure 1.3).

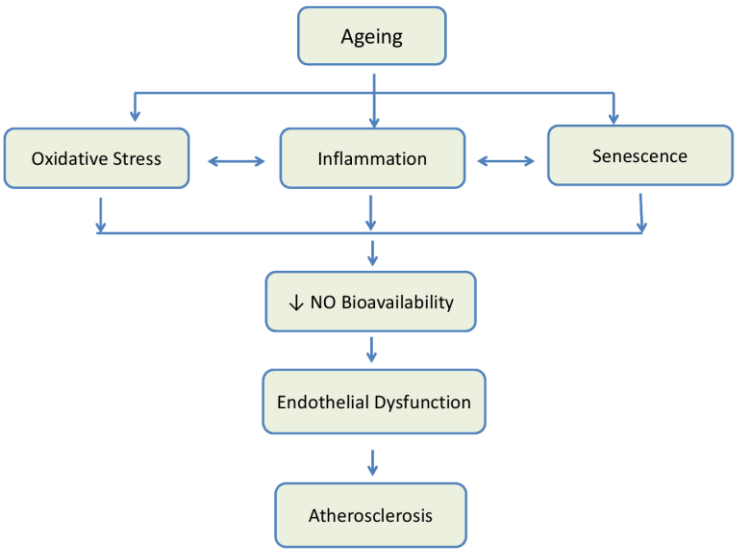


Figure 1.3: Factors associated with vascular ageing and atherosclerosis. The triad of oxidative stress, inflammation and endothelial cell senescence contribute to reduced nitric oxide (NO) availability, endothelial dysfunction and the subsequent atherosclerosis.

b. Inflammation

Chronic inflammation is a driver of ageing and contributes to the pathology of many age-related diseases including atherosclerosis (Chung *et al.*, 2009). Observational and experimental studies have demonstrated the importance of inflammation as a determinant of an unhealthy ageing phenotype. For example, the Whitehall II study reported that a high level of interleukin 6 almost halved the odds of successful ageing after 10 years (OR=0.53) and increased the risk of cardiovascular events and non-cardiovascular mortality (Akbaraly *et al.*, 2013). In addition, there appears to be a synergistic interaction between DNA damage and inflammatory signals and, in mice, chronic inflammation aggravates telomere dysfunction and cellular senescence independent of genetic and environmental factors (Jurk *et al.*, 2014). In this recent study, administration of anti-inflammatory agents rescued telomere dysfunction, prevented cellular senescence and supported tissue regenerative potential (Jurk *et al.*, 2014).

Growing evidence suggests important cross-talk between oxidative stress, inflammatory processes and the onset of ED prior to atherosclerosis (Ungvari *et al.*, 2010). Reactive oxygen species induce pro-inflammatory changes in vascular endothelium – described as "endothelial activation" - which involves secretion of autocrine/paracrine factors, leukocyte-endothelial interaction and the up-regulation of expression of cellular adhesion molecules (Herrera *et al.*, 2010).

Oxidative stress activates redox-sensitive transcription factors including the activator protein (AP-1) and nuclear factor-κB (NF-κB), increasing the expression of cytokines (TNF-α, IL-1 and IL-6), adhesion molecules (ICAM and VCAM) and pro-inflammatory enzymes (iNOS and cyclooxygenase-2) (El Assar *et al.*, 2012).

Aging is associated with higher circulating concentrations of cytokines, especially TNF-α, IL-1β and IL-6, which mediate the acute phase protein C-reactive protein (CRP). These factors contribute significantly to the pro-inflammatory microenvironment and facilitate the development of vascular dysfunction (El Assar *et al.*, 2012). Among middle-aged and older adults, the Framingham Heart Study showed that brachial FMD is inversely related to CRP, IL-6 and ICAM inflammatory markers (Vita *et al.*, 2004). Furthermore, inhibition of NF-κB signalling improved EF significantly in middle-aged and older adults (Pierce *et al.*, 2009).

c. Senescence

Cellular senescence is characterized by permanent loss of mitotic capability associated with morphological and functional changes and impaired cellular homeostasis (Erusalimsky, 2009). Senescent cells are characterised by the absence of proliferative markers, by the presence of senescent-associated β-galactosidase activity, by increased expression of tumour suppressor genes and cell cycle inhibitors, by increased DNA damage markers, more nuclear foci of constitutive heterochromatin and prominent secretion of signalling molecules (Munoz-Espin and Serrano, 2014). Without functional telomeres, DNA at the ends of chromosomes would be eroded each time a cell divides i.e. during mitosis (Farsetti *et al.*, 2009). In addition to telomere length shortening, alterations in several signalling pathways contribute to the occurrence of cellular senescence (van Deursen, 2014). Most of these signalling pathways activate p53 and they converge in activation of the cyclin-dependent kinase (CDK) inhibitors p16, p15, p21 and p27 (Munoz-Espin and Serrano, 2014).

All the risk factors for atherosclerosis including oxidative stress, inflammation, smoking, diabetes and hypertension have been associated with accelerated telomere shortening (Fyhrquist and Saijonmaa, 2012). Furthermore, clinical trials showed that the presence of atherosclerosis is independently associated with increased telomere shortening (Zietzer and Hillmeister, 2014). The induction of senescence may be related to the enhanced susceptibility of telomeres to oxidative damage (Kurz *et al.*, 2004). Telomere length in endothelial cells is inversely proportional to patient age (Aviv *et al.*, 2001) and this shortening is exacerbated in elderly patients with coronary artery disease (Ogami *et al.*, 2004). Cross sectional studies demonstrate that those with increased arterial stiffness, an indicator of vascular ageing, have

shorter telomeres (Nawrot *et al.*, 2010). Hypertensive patients have shorter telomeres than their normotensive peers and hypertensives with shorter telomeres are more likely to develop atherosclerosis over five years follow-up (El Assar *et al.*, 2012). The above observations suggest that telomere length might be a good marker for cardiovascular ageing but several issues limit the interpretation of these studies. Characterisation and quantification of telomere length is difficult and not all methods are reliable. Second, the small sample size and heterogeneity of the populations included limits confidence in these studies. Lastly, the cross-sectional design of many of these studies precludes definitive conclusions particularly in the context of CVD which has multiple risk factors, which influence and confound each other (Zietzer and Hillmeister, 2014).

Vascular endothelial cell senescence *in vivo* has been observed (Minamino *et al.*, 2002). The development of more senescent endothelial cells was associated with a shift from an anti-atherosclerotic phenotype (characterised by decreased levels of NO, eNOS activity and shear stress-induced NO production) to a pro-atherosclerotic phenotype (indicated by increased reactive oxygen species, thromboxane A_2 and endothelin-1). These observations implicate endothelial cell senescence in the initiation and progression of atherosclerosis (Minamino and Komuro, 2007). Nitric oxide increases telomerase activity and promotes mobilisation of endothelial progenitors cells (EPCs) which have the potential to delay endothelial cell ageing by replacing damaged endothelial cells to maintain physical and functional integrity of the endothelium (Farsetti *et al.*, 2009).

1.9 Antioxidant vitamins as a strategy to delay vascular ageing

Antioxidants are endogenous or exogenous moieties which inhibit or delay the oxidation of biological macromolecules (proteins, lipids and nucleic acids). Endogenous antioxidant defences include both enzymatic (SOD, GPx and catalases) and non-enzymatic (uric acid, glutathione, thiols and albumin) components. Exogenous antioxidant defences include some vitamins (vitamins C and E and the pro-vitamin A molecule beta carotene) and polyphenols which are present in relatively high concentrations in plant-based foods including vegetables, fruits and wholegrain products (Fusco *et al.*, 2007).

Furthermore, the causal role played by oxidative stress in the pathogenesis of cardiovascular diseases and the, overall, positive associations between high anti-oxidant intake and lower incidence of cardiovascular diseases and mortality found in epidemiological studies have stimulated the design of large clinical trials (e.g. the Women's Health Study and the Physicians'

Health Study II) which have attempted to test the anti-oxidant hypotheses. The results of these seminal studies were not encouraging since they found no evidence for beneficial effects of supplementation with large doses of antioxidant vitamins on cardiovascular outcomes (Ridker *et al.*, 2005; Sesso *et al.*, 2008). However, follow up, secondary analyses of the same datasets and subsequent meta-analyses have led to a re-evaluation of the conclusions and have suggested that antioxidant vitamin supplementation might be beneficial only among subjects who experience a pro-oxidant state due to high oxidative damage and/or low antioxidant status, but not for the whole population (Cesari *et al.*, 2014). In other words, the sub-group of participants experiencing a pro-oxidant state might be those with the potential to benefit from enhanced antioxidant status.

Ageing is associated with increased risk of micronutrient deficiency and diet-related illnesses (Ahmed and Haboubi, 2010). In the United States, total energy intake decreases substantially with age: by 1000 kcal–1200 kcal in men, and by 600 kcal–800 kcal in women in the seventh decade (Thomas, 2006). This results in concomitant declines in intakes of most nutrients. Lower food intake among the elderly has been associated with lower intakes of calcium, iron, zinc, B vitamins and vitamin E (Thomas, 2006). In the following sections, I will focus on vitamin C as a prototype antioxidant vitamin, discussing in more detail the molecular mechanisms which may modulate the effects of vitamin C on cardiovascular ageing.

1.10 Vitamin C (ascorbic acid)

Ascorbic acid is a six-carbon compound which was isolated first in 1928. Its structure was determined in 1933 and the L-isomer is the active form. The Home sapiens is one of the few mammals unable to synthesise vitamin C (ascorbic acid). This inability to synthesise vitamin C results from the lack of the last enzyme in the pathway of vitamin C synthesis (gulonolactone oxidase). A classical set of symptoms called scurvy, now known to be due to inadequate vitamin C intake, had been known for centuries (Lykkesfeldt *et al.*, 2014). Chemically, vitamin C is an electron donor, or reducing agent. Beside its ability to reduce oxidized species, electrons from ascorbate can reduce metals such as copper and iron leading to the formation of superoxide and hydrogen peroxide; these subsequently will generate reactive oxygen species (Rietjens *et al.*, 2002). Therefore, under certain circumstances, vitamin C becomes pro-oxidant rather than antioxidant (Halliwell, 1996). Ascorbic acid loses its electrons in two stages. When the first electron is lost, the product formed is the ascorbate radical with a short half-life (seconds to minutes). Dehydroascorbic acid (DHA) is formed after losing the second electron. Both DHA and the ascorbate radical are reversibly reduced to ascorbic acid (Padayatty and Levine, 2016).

1.10.1 *Daily requirement, sources, dietary intakes and population status of vitamin C*

In foods, vitamin C is found mainly in fruits and vegetables with the highest concentrations in cantaloupe, grapefruit, kiwi, mango, orange, tangerine and strawberries. As a supplement, vitamin C is available in tablet or powder form and it is included in many multi-vitamin formulations (Padayatty *et al.*, 2003).

The US recommended daily allowance (RDA) for vitamin C is 75 mg for adult women and 90 mg for men with an upper tolerable limit of 2000mg/day for both. However, recommendations for vitamin C intake vary between countries with lower requirements set by the UK (40mg/day) and higher recommendations set by Japan's National Institute of Health and Nutrition (100mg/day). The latter level of intake is associated with plasma concentrations of vitamin C of ~70 μmol/l and, with greater intakes, circulating concentrations of vitamin C will reach, or exceed, the renal threshold of 80 μmol/L and result in increasing urinary excretion (Frei *et al.*, 2012).

Many epidemiological studies have demonstrated that a significant proportion of the Western population suffer from hypovitaminosis C (estimated as a plasma concentration less than 28 μmol/l) (Lykkesfeldt and Poulsen, 2010; Frei *et al.*, 2012). In the third National Health and Nutrition Examination Survey (NHANES III), about 10% of the included American population suffered from severe vitamin C deficiency (plasma concentration less than 11 μmol/l) (Frei *et al.*, 2012). Additionally, 20% of males and 15% of females had marginal vitamin C deficiency (determined as plasma concentrations between 11-28 μmol/l) (Schleicher *et al.*, 2009). The Low Income Diet and Nutrition Survey (2003-05) of a representative sample of the low-income/materially deprived UK population estimated that 25% of men and 16% of women had deficient plasma vitamin C concentrations (Mosdol *et al.*, 2008). In the third Glasgow MONICA study (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease), 26% of men and 14% women were in the severe vitamin C category (Wrieden *et al.*, 2000). Moreover, in the French population, 7 to 12 % of males and 3 to 5% of females suffered from severe vitamin C deficiency while 10 to 46% of males and 3 to 15% of females categorised with marginal vitamin C deficiency (Lykkesfeldt and Poulsen, 2010).

The age-related increase in vitamin C deficiency may be due to a decline among older people in the capacity to absorb vitamin C as a consequence of reduced activity of sodium-dependent vitamin C transporter proteins in the intestine. This reduction in vitamin C absorptive capacity is associated with chronic reduction in vitamin C concentration in the elderly (Visioli and Hagen, 2007). A meta-analysis of the relationship between vitamin C intake and plasma

concentration of vitamin C found that, at the same intake (60 mg/d), circulating concentrations were 25% less among older adults (>65 years) than in younger adults (Brubacher *et al.*, 2000). The authors concluded that the elderly would require double the oral intakes of vitamin C, as compared with younger subjects, to maintain plasma concentration of 50 µmol/l (Brubacher *et al.*, 2000; Li and Schellhorn, 2007).

1.10.2 ***Cardiovascular effects of vitamin C***

An overview of the pathways linking vitamin C and cardiovascular ageing is provided in Figure 1.4.

a. Antioxidant effect

Vitamin C can be oxidised by free radicals, reactive oxygen species and compounds that react with free radicals and which are changed to radicals themselves such as tocopheroxyl radicals (Duarte and Lunec, 2005). Vitamin C counteracts the potential damaging effects of oxidative stress through its ability to quench aqueous reactive oxygen and nitrogen species and by helping to regenerate the antioxidant vitamin E (Aguirre and May, 2008). In addition, vitamin C may mitigate the earliest stages of atherosclerosis through the following mechanisms: 1) preventing the oxidation of LDL and reducing uptake and modification by activated macrophages; 2) stimulation of endothelial cell proliferation and prevention of apoptosis; 3) decreasing recruitment and proliferation of vascular smooth muscle cells and 4) increasing type IV collagen synthesis (Aguirre and May, 2008).

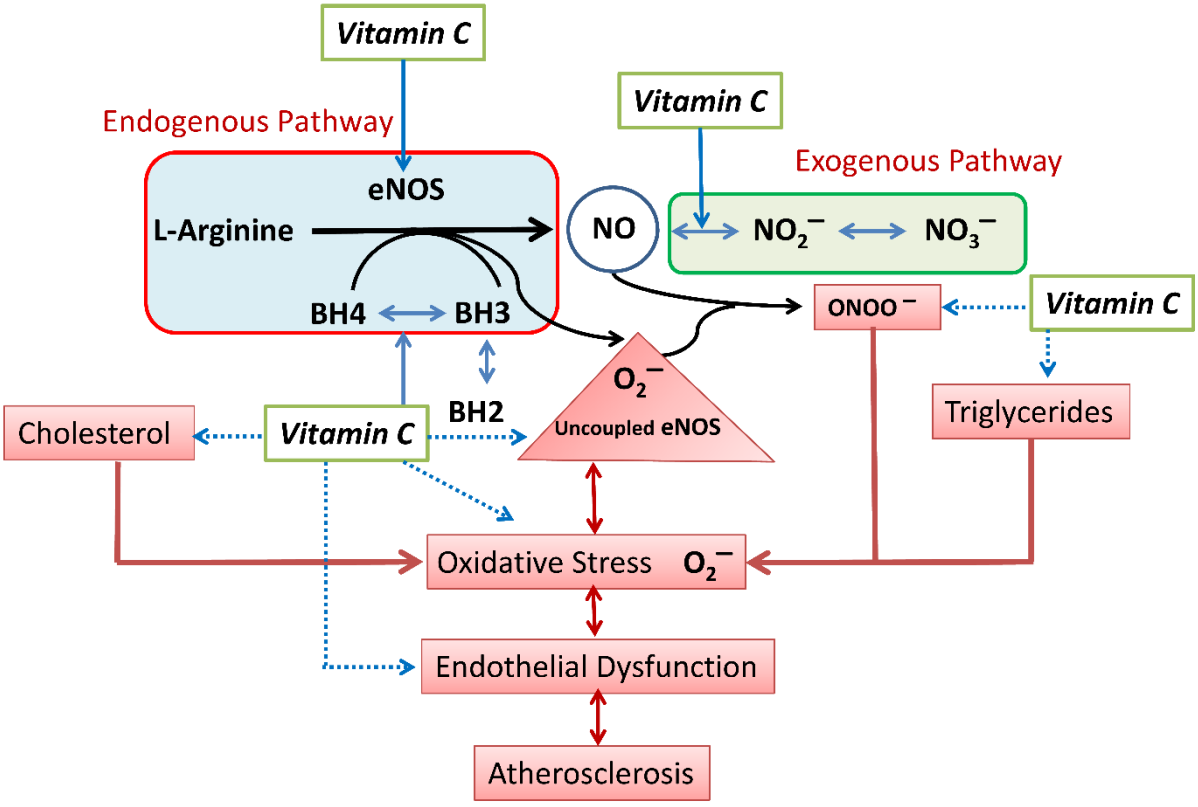


Figure 1.4: Vitamin C enhances nitric oxide (NO) bioavailability by affecting both the internal (L-arginine, endothelial nitric oxide synthase [eNOS]) and external pathways (NO_3^-- NO_2^-- NO) of NO synthesis. Dotted lines represent inhibition while continuous lines represent stimulation. BH4, tetrahydrobiopterin, $ONOO^-$, peroxynitrite.

b. Nitric oxide-sparing effect

Vitamin C contributes to maintenance of the total pool of NO through several different mechanisms. (The NO pool represents the released NO which come in direct contact with the blood and can also be transported throughout the body to function in a paracrine fashion, much like a hormone (Rassaf *et al.*, 2004)). 1) Superoxide free radicals are highly reactive molecules which, in excess, may react with NO leading the formation of the harmful free radical peroxynitrite. Vitamin C inactivates the superoxide free radical leading to higher bioavailability of the protective NO (May, 2000). 2) Vitamin C stabilizes, and increases the synthesis of BH4, a cofactor which is essential for the function of eNOS. BH4 deficiency leads to eNOS uncoupling and the production of superoxide instead of NO (Schmidt and Alp, 2007). 3) Inhibition of the enzyme arginase and consequent increase in L-arginine bioavailability (May, 2000). 4) Vitamin C may enhance the activity of eNOS by acting as a cofactor in hydroxylation reactions that lead to NO release. In addition, vitamin C may protect the eNOS enzyme from S-nitrosylation and, consequently, inactivation (Holowatz, 2011). 5) Vitamin C may enhance

the functional activity of NO by preserving guanylate cyclase activity and, hence, the formation of cGMP, the second messenger of NO production (May and Harrison, 2013). 6) Vitamin C-induced enhanced decomposition of nitrosothiol into NO (May, 2000). 7) Enhancing the NO_3^--NO_2^--NO pathway. In vitro and animal studies have shown that vitamin C may convert NO_2^- to NO in the gastrointestinal tract and plasma leading to increased availability of NO in the tissues (Wolff and Wasserman, 1972; Mowat and McColl, 2001).

c. Lipid-lowering effect

Vitamin C facilitates the conversion of cholesterol into bile acids by modifying the activity of the rate-limiting enzyme cholesterol 7a-hydroxylase involved in bile acid synthesis and, therefore, may help to lower blood cholesterol concentrations. Animals deficient in vitamin C exhibit hypercholesterolemia and the subsequent administration of vitamin C restores cholesterol concentrations to normal (Chambial *et al.*, 2013). Furthermore, human observational studies showed that high plasma concentrations of vitamin C were associated with more favourable lipid profiles (Dobson *et al.*, 1984; Khaw *et al.*, 2001) but whether this is a causal relationship is uncertain. A meta-analysis of 13 trials which recruited participants with hypercholesterolemia showed that vitamin C supplementation reduced concentrations of LDL-C (weighted mean difference [WMD]: -7.9 mg/dL; 95% CI: -12.3 to -3.5) and triglycerides (WMD: -20.1 mg/dL; 95%CI: -33.3 to -6.8) significantly with no significant effect on HDL-C (WMD: 1.1 mg/dL; 95% CI: -0.2 to 2.3) concentrations (McRae, 2008).

d. Blood pressure-lowering effect

A population-based cross-sectional study involving 20,926 participants with mean age of 58.5 years demonstrated that people in the highest quartiles of plasma vitamin C concentration had lower clinic blood pressure (odds ratio: 0.78; 95% CI: 0.71 to 0.86) in comparison with the bottom quartiles after adjusting for other relevant factors (Myint *et al.*, 2011). Block *et al.* (2001) conducted a study involved 68 men aged 30 to 59 years who had a mean systolic BP of 122.2 mmHg and a mean diastolic BP of 73.4 mm Hg. The participants were subjected to one month depletion/one month repletion of vitamin C and the investigators found that individuals in the lowest quartile of plasma ascorbic acid had >7 mm Hg higher diastolic blood pressure than did those in the top quartile of the plasma ascorbate. A recently published systematic review and meta-analysis has shown that vitamin C can lower systolic BP and diastolic BP significantly. The pooled changes in systolic BP and diastolic BP were -3.84 mm Hg and -1.48 mm Hg respectively. The proposed mechanism is the ability of vitamin C to reduce oxidative stress and to increase NO availability (Juraschek *et al.*, 2012).

1.10.3 ***Prooxidant effects of vitamin C***

Vitamin C may act as prooxidant rather than as an antioxidant in high pharmacological doses, high oxygen tension and in the presence of metal ions (Halliwell, 1996; Rietjens *et al.*, 2002). For example, intraperitoneal injection of 4 g/kg of vitamin C to mice resulted in a significant increase in the formation of both ascorbate radicals and H_2O_2 (Chen *et al.*, 2007). *In vitro*, vitamin C can convert Fe^{3+} into Fe^{2+}, which then reacts with oxygen or hydrogen peroxide resulting in the formation of superoxide anions and hydroxyl radicals (Rietjens *et al.*, 2002). This iron/ascorbate mixture has been used as a model of lipid peroxidation stimulation. Adding vitamin C to purified DNA in the presence of redox active metal ions causes single-strand breaks and base modifications such as 8-oxo-2-deoxyguanosine (8-oxodG) (Halliwell, 1996). In contrast, vitamin C intake in the dose range of 150-900 mg/kg did not appear to affect lipid peroxidation in rats (Halliwell, 1996). In guinea pigs initially subjected to marginal vitamin C deficiency, optimum and mega-doses of vitamin C had no effect on the levels of 8-oxodG (Carr and Frei, 1999). In healthy human volunteers aged 17 and 49 years, six weeks of supplementation with 500mg vitamin C per day produced a significant reduction in 8-oxoguanine and a significant increase in 8-oxoadenine (Podmore *et al.*, 1998). However, *post hoc* analysis of serum and urine samples from the above study demonstrated significant reduction in the concentration of 8-oxodG (Cooke *et al.*, 1998). Moreover, Rehman *et al.* (1998) showed significant increase in total oxidative DNA base damage with the co-administration of iron and vitamin C to participants with relatively high baseline plasma concentration of vitamin C (> 71.9µmol/L). However, further work is needed to investigate the pro-oxidant effects of vitamin C supplementation in long-term clinical trials.

1.10.4 ***Genetic influences on vitamin C status and impact on cardiovascular risk***

Vitamin C homeostasis is influenced by several common genetic variants (Michels *et al.*, 2013). These genetic variations fall into two major categories. The first category are variants in genes which encode proteins responsible for vitamin C absorption and transport, the sodium-dependent vitamin C transporters 1 and 2 (SVCT1, SVCT2) which are encoded by the SLC23A1 and SLC23A2 genes respectively. SVCT1 is expressed in epithelial cells, notably the enterocytes of the small bowel where it transports vitamin C into the body. SVCT2 is expressed widely throughout the body especially in metabolically active cells. The second category of genes are those related to the antioxidant and redox activities of vitamin C and include variants in genes affecting iron homeostasis and oxidative stress such as haptoglobin and the glutathione S-transferases (GSTs) (Michels *et al.*, 2013). Transcriptional regulation of

the SLC23 genes controls the tissue distribution of vitamin C transporters (SVCTs) and is responsible for the maintenance of vitamin C concentrations in cells, tissues and extracellular fluids (Burzle and Hediger, 2012).

1.11 Inorganic NO_3^- and vascular ageing

Throughout this introduction, I have emphasised the importance of NO for a healthy endothelium and described the pivotal role of NO in the prevention of atherosclerosis. In this section, I will discuss the impact of ageing on NO bioavailability and the role of inorganic NO_3^- in restoring NO sufficiency and in improving EF in advanced age groups.

1.11.1 *Ageing-associated NO insufficiency*

Vascular NO insufficiency in older people is mediated in part by decreased NO production by eNOS (Cau *et al.*, 2012). There is evidence that eNOS activity is reduced with age because of post-translational modification such as acylation, nitrosylation, glycation or phosphorylation (Cau *et al.*, 2012). Additionally, this reduction in eNOS activity might be secondary to the deficiency of cofactors required in the process of NO production (e.g. BH4) (Seals *et al.*, 2014). The age-associated increase in arginase activity may compete with eNOS for the critical substrate required in NO production, L-arginine (Seals *et al.*, 2014). Lastly, excessive superoxide production with age may contribute to NO insufficiency. The interaction of superoxide with NO produces a highly reactive nitrogen species, peroxynitrite (Sindler *et al.*, 2014). Due to its ability to restore reduced NO bioavailability, inorganic NO_3^- represents a potential therapeutic strategy to treat age-associated vascular dysfunction (Sindler *et al.*, 2014).

1.11.2 *A historical perspective of the* NO_3^- - NO_2^- - NO *pathway*

Many epidemiological studies have shown apparently protective effect of higher intakes of fruits and vegetables against cardiovascular diseases. On the assumption that the beneficial effects of fruits and vegetables were due to their contents of antioxidant vitamins (Kevil *et al.*, 2011), many RCTs of supplemental antioxidant vitamins were launched. Unfortunately, large scale clinical trials of such supplementation have not revealed beneficial effects of these antioxidants vitamins on cardiovascular outcomes (Hord *et al.*, 2009).

Until that time, NO_3^- and NO_2^- contents of fruits and vegetables were thought to be inert substances or even harmful to the human body. Inorganic NO_3^- was linked to the notorious substances nitrosamines accused of causing cancer in animal studies and the intake of NO_3^-

was strictly regulated (Kapil *et al.*, 2010b). Vegetables are the dominant source of inorganic NO_3^- in diet (> 80%) and leafy green vegetables, in particular, contain a high amount of this anion. Furthermore, the beneficial BP lowering effect of the DASH diet may, in part, be due to its high contents of inorganic NO_3^- (Hord *et al.*, 2009).

It has been believed that the beneficial effect of NO disappears in a few seconds as it is converted to NO_2^- and then to NO_3^-. NO_2^- and NO_3^- are then excreted in urine indicating the end of the story for the biological effects of NO. Interestingly, in the last two decades, scientists discovered an alternative pathway for generating NO other than the classical L-arginine-eNOS-NO pathway (Zweier *et al.*, 1995). The other source of NO was found to be NO_2^- which can be converted back to NO by the action of several enzymes and molecules including deoxyhaemoglobin, deoxymyoglobin, xanthine oxidoreductase, protons, polyphenols and ascorbic acid. Of note, this pathway is more active and efficient in cases of hypoxia in which the level of both oxygen and NO are low (Zweier *et al.*, 1999) (Figure 1.5).

1.11.3 ***Dietary sources of inorganic NO_3^-***

Eighty five per cent of the dietary NO_3^- is derived from vegetables and the remaining is mostly from drinking water. Our dietary intake of NO_2^- comes from cured meat, to which it is added to prevent the development of botulinum toxin. Vegetables can be categorised into three categories according to their NO_3^- content: 1) high NO_3^- contents, e.g. rocket, spinach, lettuce and beetroot (> 1000 mg/kg); 2) medium NO_3^- contents, e.g. turnip, cabbage, green beans, cucumber and carrot (100-1000 mg/kg); 3) low NO_3^- contents, e.g. onion and tomato (< 100 mg/kg) (Lidder and Webb, 2013). The concertation of NO_3^- in drinking water varies according to geographical location and regional rules regarding safe levels of NO_3^- in tap or bottled water. The permissible concentration of NO_3^- in drinking water is 50 mg/L in Europe and 44 mg/L in the US according to the WHO recommendations (Bryan and Ivy, 2015).

The acceptable daily intake for NO_3^- set by the European Food Safety Authority (EFSA) is 3.7 mg/kg (0.06 mmol/kg). Those consuming a vegetarian diet have been found to ingest 4.3 mmol NO_3^- per day which is 4 times higher than the intake of omnivores and greater than the EFSA recommendation of ~ 1.2 mmol NO_3^- per day (Lidder and Webb, 2013).

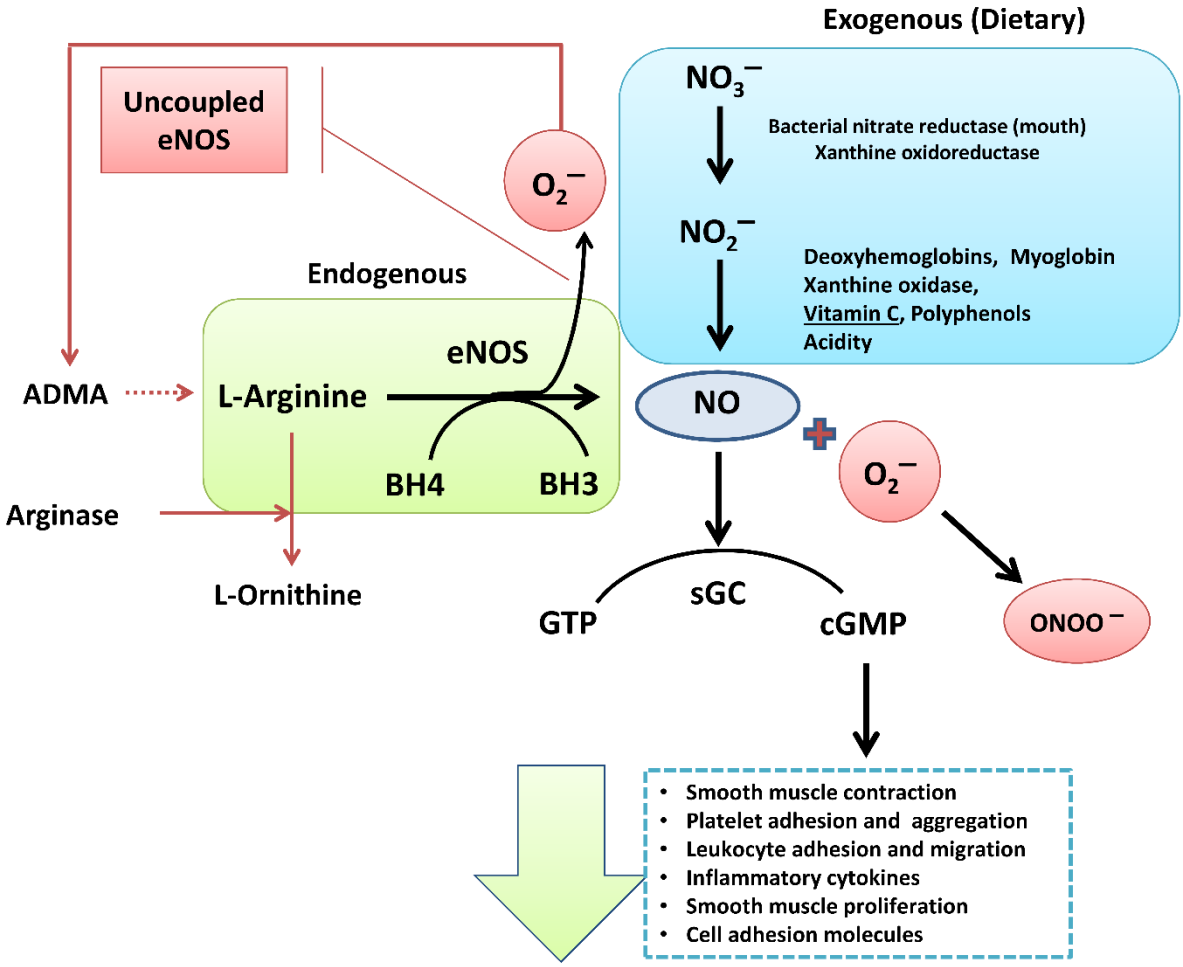


Figure 1.5: Exogenous and endogenous sources of nitric oxide (NO). NO is produced by a family of enzymes known as NO synthases (NOS) which utilize the substrate L-arginine. The NO_3^-–NO_2^-–NO pathway has been proposed as an alternative pathway for NO generation. ADMA: asymmetric dimethylarginine; BH4: tetrahydrobiopterin; cGMP: cyclic guanosine monophosphate; GTP: guanosine triphosphate; $ONOO^-$, peroxynitrite; sGC: soluble guanylate cyclase.

1.11.4 *Entero-salivary circulation of* NO_3^-

Dietary NO_3^- is well-absorbed in the upper gastrointestinal tract with approximately 100% bioavailability and plasma concentration of NO_3^- peaking after 1 hr (Lidder and Webb, 2013). About 25% of the circulating pool of NO_3^- is actively taken up from the blood via an anion exchange channel called sialin and secreted by the salivary glands into the saliva (Bailey *et al.*, 2014). The salivary NO_3^- is reduced to NO_2^- by facultative anaerobic bacteria in the oral cavity. This NO_2^- and other inorganic NO_3^- travel to the stomach where they are converted to NO under the high acidity of the stomach and with the help of ascorbic acid (Hord *et al.*, 2009). In this strong acidic environment of the stomach, NO_2^- is protonated to form nitrous acid (HNO_2) (Bailey *et al.*, 2014). Nitrous acid can spontaneously give rise to the generation of NO

through the following sequence of reactions: $2HNO_2 \rightarrow H_2O + N_2O_3$ and $N_2O_3 \leftrightarrow NO + NO_2$ (Butler and Feelisch, 2008). The liberated NO has been found to be protective for the gastric mucosa (enhances blood supply). Moreover, the remaining NO, NO_2^-, and NO_3^- diffuse to the general circulation and contribute to the NO pool (Lundberg *et al.*, 2006). Stable nitrogen isotope tracer studies demonstrated that 60% of oral NO_3^- dose is excreted via the kidneys within 48 hours (Bailey *et al.*, 2014).

1.11.5 *Mechanisms of NO_2^- reduction to NO in the circulation*

In the circulation, NO_2^- may function as a source of NO that is activated in hypoxia and acidic conditions to increase blood flow and regulate blood pressure (Zweier *et al.*, 2010; Kevil *et al.*, 2011). There are many mechanisms involved in the bioconversion of NO_2^- to NO in the blood. The most common is the reaction of deoxyhaemoglobin ($HbFe^{2+}$) with NO_2^- in an acidic environment which will liberate NO ($NO_2^- + HbFe^{2+} + H^+ \rightarrow NO + HbFe^{3+} + OH^-$) (Kim-Shapiro and Gladwin, 2014). In addition to $HbFe^{2+}$, there are many enzymes and proteins that enhance the conversion of NO_2^- to NO such as myoglobin, cytochrome C oxidase, eNOS and xanthine oxidoreductases (Kim-Shapiro and Gladwin, 2014).

1.11.6 *Therapeutic effects of inorganic NO_3^-*

NO_3^- has been used in the treatment of cardiovascular diseases (angina and digital ischemia) since medieval times according to the translation of Buddhist manuscripts (Butler and Feelisch, 2008; Machha and Schechter, 2012). In the last thirty years, since the discovery of the NO_3^--NO_2^--NO pathway, there has been a renewal in using NO_3^- and NO_2^- in experiments and in clinical trials. This discovery demonstrated the contribution of NO_3^-/NO_2^- to the overall NO pool (Kapil *et al.*, 2014).

Larsen *et al.* (2006) reported a pioneer study that demonstrates the beneficial effect of inorganic NO_3^- in BP reduction. In that study, the investigators administered 0.1 mmol sodium NO_3^-/kg body weight/day to healthy participants which corresponds to an intake of 100-300 g of NO_3^--rich vegetables per day. After three days of NO_3^- supplementation, a 4 mmHg reduction in diastolic BP was observed (Larsen *et al.*, 2006). Administration of the same dose of NO_3^- to a greater group of individuals produced significant reductions in both systolic BP and diastolic BP (Larsen *et al.*, 2007). Four weeks of NO_3^- supplementation (9 mg/kg) to older people at higher CVD risk significantly lowered systolic BP by 8 mmHg in comparison with placebo (Rammos *et al.*, 2014). Supplementing beetroot juice (providing a NO_3^- dose of 300-400 mg)

to older overweight, but otherwise healthy, participants for three weeks lowered daily home-measured systolic BP by 7 mmHg (Jajja *et al.*, 2014). In the latter study BP values returned to pre-intervention values, one week after stopping the beetroot supplementation.

In contrast, studies in diabetics or treated hypertensive patients did not show significant improvement of BP with beetroot administration (Gilchrist *et al.*, 2013; Bondonno *et al.*, 2015b). Moreover, an individual participant meta-analysis (85 participants) showed that beetroot supplementation lowered 24 hrs-ambulatory BP significantly in younger participants only (<65 years) (Siervo *et al.*, 2015). However, data synthesis from 16 studies involving 254 participants demonstrated a significant reduction of systolic BP (-4.4 mm Hg) with inorganic NO_3^- or beetroot consumption (Siervo *et al.*, 2013).

The discovery of the contribution of dietary NO_3^- to NO bioavailability has provided a rationale for the use of NO_3^- to reverse ED following NO insufficiency (Machha and Schechter, 2012). Inorganic NO_2^- supplementation reversed ED significantly in a murine model of hypercholesterolemia (Stokes *et al.*, 2009). In addition, NO_2^- supplementation reversed age-associated ED, oxidative stress and arterial stiffness in mice (Sindler *et al.*, 2011). In humans, less consistent results were observed (Webb *et al.*, 2008; Gilchrist *et al.*, 2013). Acute ingestion of inorganic NO_3^- (8 mmol) had no significant effect on FMD three hours post-intervention in healthy participants (Bahra *et al.*, 2012). Two weeks of beetroot intake by type 2 diabetics was ineffective on EF (macro- and microvascular function) (Gilchrist *et al.*, 2013). Three weeks supplementation with beetroot juice in older overweight healthy subjects had no significant effect on skin microvascular blood flow (Ashor *et al.*, 2015a). On the contrary, a recently conducted study in 15 young healthy participants revealed a noticeable improvement in FMD after acute ingestion of inorganic NO_3^- (Rodriguez-Mateos *et al.*, 2015) and six weeks daily intake of beetroot juice improved FMD significantly in patients with hypercholesterolemia (Velmurugan *et al.*, 2016).

Inorganic NO_2^- reversed ageing-related arterial stiffness in older mice. In the latter study, Sindler *et al.* (2011) observed that plasma NO_2^- concentration was reduced in older mice and that this was restored to youthful concentrations with inorganic NO_2^- supplementation. In healthy humans, Bahra *et al.* (2012) observed a significant reduction in arterial stiffness three hours after NO_3^- ingestion. Moreover, daily consumption of NO_3^- (900 mg) for four weeks reduced pulse wave velocity in older people at increased CVD risk (Rammos *et al.*, 2014). However, arterial compliance increased with no change in pulse wave velocity after 220 mg NO_3^- supplementation in 28 healthy participants (Liu *et al.*, 2013).

In vitro studies have shown that inorganic NO_3^- prevents the oxidation of LDL and so may slow down the process of atherogenesis (Kapil *et al.*, 2010b). In addition, feeding inorganic NO_3^- to mice with hypercholesterolemia reduced circulating triglyceride concentrations (Stokes *et al.*, 2009). Moreover, inorganic NO_3^- administration inhibits platelet aggregation and, therefore, may reduce thrombotic events in both humans and experimental animals (Richardson *et al.*, 2002; Park *et al.*, 2013).

The restoration of blood to a tissue after a period of ischemia is sometimes associated with severe tissue injury due to a high release of free radicals. Animal studies have demonstrated that the prior administration of inorganic NO_3^- reduces the infarct size in a model of ischaemic-reperfusion injury (Lundberg *et al.*, 2011). Moreover, low dose sodium NO_2^- attenuated myocardial ischemia and vascular reperfusion injury in a human experimental study (Ingram *et al.*, 2013).

Administration of inorganic NO_3^- is associated with reduced oxygen consumption during exercise which has been interpreted as an increase in the energetic efficiency of exercise (Jones, 2014). It has been proposed that NO increases the efficiency of oxidative phosphorylation by reducing the leak of protons from the mitochondria (Kapil *et al.*, 2010b; Lundberg *et al.*, 2011). Whilst dietary NO_3^- supplementation increased exercise performance in most studies with young healthy participants (Hoon *et al.*, 2013), similar studies in older individuals or diseased patients revealed contradictory results (Kelly *et al.*, 2013; Shepherd *et al.*, 2015).

Data from animal studies have shown promising results regarding the effect of dietary NO_3^- on biomarkers of metabolic diseases. Supplementation of eNOS-deficient mice suffering metabolic syndrome with inorganic NO_3^- for 10 weeks reduced visceral fat and circulating triglycerides concentration and reversed the pre-diabetic phenotype (Carlstrom *et al.*, 2010). Furthermore, supplementing diabetic rats with sodium NO_3^- for two months produced significant improvements in glucose homeostasis, lipid profile and oxidative stress markers (Khalifi *et al.*, 2015). However, NO_3^- supplementation in humans did not result in improvement in glucose and insulin homeostasis in diabetic (Gilchrist *et al.*, 2013; Cermak *et al.*, 2015; Shepherd *et al.*, 2015) or in non-diabetic participants (Larsen *et al.*, 2014).

1.12 Vitamin C and inorganic NO_3^- interaction

Epidemiological studies have associated higher intakes of vitamin C with protective effects from nitrate-associated adverse events such as colorectal cancer and diabetes mellitus (Dellavalle *et al.*, 2014; Bahadoran *et al.*, 2017). The purported mechanisms of the beneficial

effects of vitamin C include the wash-out of NO_2^- by converting it into NO and, therefore, prevention of the formation of NOCs (Mowat *et al.*, 1999). Vitamin C generate NO over a wide pH range in aqueous solution by rapid reduction of nitrous acid and production of DHA (Bartsch *et al.*, 1988).

Acidification of urine and ingestion of vitamin C has been used in the treatment and prophylaxis against urinary tract infection. Ingestion of 2 g of vitamin C over 2 days induced a sevenfold increase in NO release from nitrite-containing urine from healthy individuals (Lundberg *et al.*, 1997). Vitamin C may enhance the conversion of NO_2^- into NO in the circulation, therefore, further contributing to the NO pool (Lundberg *et al.*, 2008).

In the previous sections, we have demonstrated that both inorganic NO_3^- and vitamin C, separately, may enhance NO production and improve EF. Moreover, the co-administration of vitamin C with inorganic NO_3^- may further enhance the conversion of NO_2^- to NO, therefore an additive or synergistic effect on EF might be expected from this combination. Synergism means that the combined effect of both dietary agents is greater than that predicted by their individual potencies (Tallarida, 2011). The additive effect is the situation in which the effect produced by the dietary agents is the algebraic sum of their independent actions. A synergistic interaction allows the use of lower doses of dietary agents, a situation that may reduce adverse reactions (Tallarida, 2001).

1.13 Hypotheses, aims and objectives

The hypotheses for this project were:

1. Dietary NO_3^- supplementation will improve EF and arterial stiffness in human adults. The effect on vascular function will be associated with a significant reduction in blood pressure. Moreover, inorganic NO_3^- supplementation will ameliorate the effects of hyperglycaemia on skin microvascular blood flow and biomarkers of EF in young and older obese individuals.
2. Vitamin C supplementation will improve EF and arterial stiffness in adult humans. This effect will be accompanied by a reduction in blood pressure.
3. Combined inorganic NO_3^- and vitamin C supplementation will have a greater effects on blood pressure, skin microvascular blood flow and arterial stiffness than each intervention alone in both young and older healthy participants.

The aims of this thesis were to test the above hypotheses by investigating the effects of inorganic NO_3^- and vitamin C supplementation (alone or in combination) on blood pressure and EF. Furthermore, I investigated whether the effects of these interventions were different between younger and older participants.

The specific objectives of the study included:

1. To carry out systematic reviews and meta-analyses to examine the evidence for effects of inorganic NO_3^-/beetroot supplementation on EF.
2. To carry out systematic reviews and meta-analyses to examine the evidence for acute and chronic effects of vitamin C on biomarkers of CVD.
3. To undertake an intervention study to investigate the acute effects of inorganic NO_3^- on EF in young and older obese healthy volunteers subjected to acute hyperglycaemia.
4. To undertake an intervention study to investigate the effects of vitamin C and of inorganic NO_3^- alone, and in combination, on blood pressure and vascular function in young and older healthy volunteers.

Chapter 2. The effects of inorganic NO_3^- and beetroot supplementation on EF: An updated systematic review and meta-analysis

2.1 Introduction

NO is the key molecule secreted from endothelial cells which regulates vascular tone, platelet aggregation, thrombosis, monocytes adhesion and smooth muscle proliferation (Forstermann, 2010). Endothelial dysfunction appears in the early stages of the pathogenesis of vascular disorders and it is closely related to the progression of severe clinical conditions (i.e. stroke, pulmonary embolism and myocardial infarction) (Pober *et al.*, 2009). Endothelial dysfunction is also commonly observed in metabolic disorders associated with increased atherosclerotic risk such as diabetes mellitus, metabolic syndrome and hypercholesterolemia (Singhal, 2005; Tang and Vanhoutte, 2010; Sena *et al.*, 2013).

The imbalance created by over-production of pro-inflammatory free radicals and reduced bio-availability of NO alters EF and activates mechanisms (i.e. vasoconstriction, monocyte activation, smooth muscle cell proliferation and hyper-coagulation) which are causally linked to the formation of atherosclerotic plaques (Forstermann, 2010).

Arterial stiffening is a hallmark of ageing and is closely associated with many pathological conditions including atherosclerosis, dyslipidaemia, diabetes and chronic kidney diseases (Shirwany and Zou, 2010). Reduced arterial compliance (i.e. increased stiffness) leads to faster reflection of the systolic wave from the peripheral small arteries to the heart, causing augmentation of the central aortic pressure (Cavalcante *et al.*, 2011). This augmentation in central pressure leads to increased ventricular afterload and reduced coronary perfusion pressure which, eventually, may cause myocardial hypertrophy, ischemia and infarction (Sakuragi and Abhayaratna, 2010). Thus, arterial stiffness appears to contribute to the complex aetiology of CVDs and is regarded as a predictor of increased CVD risk and all-cause mortality (Laurent and Boutouyrie, 2007).

Numerous nutritional interventions that target the conventional NO pathway have been proposed to maintain or restore healthy endothelial reactivity (Bai *et al.*, 2009; Hall, 2009; Hord, 2011; Lovegrove and Griffin, 2013). For example, supplementation with L-arginine, the substrate for constitutive NO synthase that governs tonic endothelial NO generation, has been assessed in healthy individuals and in multiple disease states but with mixed results and the long-term benefits of arginine supplementation have been questioned (Luiking *et al.*, 2012).

Similarly, provision of co-factors for NOS activity, particularly tetrahydrobiopterin (Alp and Channon, 2004), improved EF in healthy and specific cohorts of individuals with CVD (Bendall *et al.*, 2014). However, tetrahydrobiopterin demonstrated no effects on EF in patients with coronary artery disease (Cunnington *et al.*, 2012; Bendall *et al.*, 2014).

More recently, considerable interest has focussed on elevating NO utilising pathways independent of the conventional L-arginine/NO synthase pathway. Dietary NO_3^- supplementation is one of these pathways that is regarded as an external source of nitric oxide (Lidder and Webb, 2013). Benjamin (Benjamin *et al.*, 1994) and Lundberg (Lundberg *et al.*, 1994) separately demonstrated that administration of oral NO_3^- to healthy volunteers resulted in elevations of gastric NO_2^- that upon entry into the low pH environment of the stomach was converted to NO. Later, in a double-blind crossover study, Larsen et al. reported, for the first time, reduction in diastolic BP (-3.7 mmHg) after sodium NO_3^- consumption by healthy volunteers (Larsen *et al.*, 2006).

Subsequently, studies confirmed the effects of NO_3^- or beetroot (juice) on blood pressure (Siervo *et al.*, 2013). Moreover, inorganic NO_3^- or beetroot prevented endothelial dysfunction (measured by brachial artery FMD induced by an acute ischemic insult in the human forearm and significantly attenuated *ex vivo* platelet aggregation in response to collagen and adenosine diphosphate (Webb *et al.*, 2008). Importantly, the BP-lowering and vascular effects of inorganic NO_3^- may be due to the endogenous conversion of NO_3^- to NO (Gladwin *et al.*, 2005; Lundberg *et al.*, 2009).

It is plausible that the BP lowering effects of dietary inorganic NO_3^- intake may in part be due to the improvements in vascular function (Hord, 2011); however a critical analysis of the evidence on this subject is lacking. Here, we conducted a systematic review and meta-analysis of the evidence from RCTs investigating the efficacy of inorganic NO_3^- and beetroot supplementation, chosen as a rich source of inorganic NO_3^-, on physiological measures of EF including FMD, FBF, microvascular blood flow, PWV, and AIx (Tousoulis *et al.*, 2005). We also investigated whether the effect of NO_3^- on EF was modified by the age, health status, baseline BMI and baseline systolic BP or diastolic BP of the included participants or modified by the daily dose or duration of inorganic NO_3^-/beetroot supplementation.

2.2 Methods

This systematic review was conducted according to the Cochrane guidelines and is reported according to PRISMA guidelines (Higgins and Green, 2008; Liberati *et al.*, 2009).

2.2.1 ***Data sources***

This systematic review and meta-analysis is an update of a previously conducted study performed by our group (Lara *et al.*, 2015). The search for relevant studies was conducted via electronic search of three databases (Medline, Embase and Scopus). Additionally, we searched for eligible studies in the reference lists of the relevant articles and reviews. The following keywords were used to search the above databases from January 2014 until January 2016: inorganic nitrate, dietary nitrate, nitrate, beetroot, endothelial function, flow mediated dilation (FMD), blood flow, vascular function, microvascular function, microcirculation, pulse wave, arterial stiffness, arterial compliance and augmentation index.

2.2.2 ***Study selection***

The following criteria were applied to identify the articles to be included in this systematic review and meta-analysis: 1) RCTs (no 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) inorganic NO_3^- or beetroot administered alone i.e. not combined with other drugs or nutritional interventions; 4) studies were not excluded because of the dose or duration of administration of NO_3^-; 5) studies reporting changes in EF measured using ultrasound, VOP, PWV, iontophoresis, pulse amplitude tonometry. Two investigators 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 article was 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 reviewers until consensus was reached.

2.2.3 ***Data extraction***

The following information was extracted from the eligible articles:

1. Authors, journal details and year of publication
2. Participants' characteristics (age, sex, health status, BMI, systolic BP and diastolic BP)
3. Study characteristics (design, sample size and study quality)
4. Inorganic NO_3^- or beetroot intervention (dose, duration and type of intervention)
5. Circulating concentrations of NO_3^- before and after intervention.

2.2.4 ***Statistical analyses***

As stated above, several methods are used to assess EF including FMD, FBF, microvascular blood flow, PWV and AIx. However, we considered for meta-analysis only outcomes reported in ≥ 5 studies (Higgins and Green, 2008). The other outcomes of < 5 reports in the studies were included in the qualitative analysis only. Therefore, the outcome of the meta-analysis is the net difference between the intervention and control group in FMD, PWV and AIx at the end of the study. The FMD was calculated by subtracting the peak diameter after hyperaemia from the baseline diameter divided by the baseline diameter and multiplied by 100 (Tousoulis *et al.*, 2005). PWV is defined as the speed of travel of the pressure pulse along the arterial segment, is calculated as the distance/transit time ratio and is expressed as meters per second (Zoungas and Asmar, 2007). The AIx is defined as the proportion of central pulse pressure due to the late systolic peak which, in turn, is attributed to the reflected pulse wave and is expressed as percentage (Yasmin and Brown, 1999).

Statistical analyses were performed using STATA 12 (StataCorp. 2011. College Station, TX, USA). The effect size (the magnitude of effect e.g. the difference between treatment mean and control mean) was estimated as weighted mean differences (WMDs) with 95% confidence interval. Random effect models were used to take account of between-study heterogeneity for participant characteristics, study design and methods used to assess endothelial function. Data not provided in the main text or tables were extracted from the figures. For crossover trials, we used the mean and SD separately for the intervention and control conditions (Elbourne *et al.*, 2002).

Subgroup analyses were undertaken to investigate the role of potential factors influencing the effect of NO_3^- on EF and accounting for the heterogeneity of the models. These factors included participants' characteristics such as health status, age of participants, baseline values for BMI, blood pressure and factors related to study characteristics including type of intervention, study design and study quality. Meta-regression analyses were conducted to examine possible relationships between the effect of NO_3^- supplementation on markers of EF (FMD, PWV and AIx) and continuous variables reported such as age, BMI, systolic BP, diastolic BP and dose and duration of NO_3^- intervention.

2.2.5 ***Quality assessment***

Indices of study quality were assessed by the modified Jadad score (range 0 – 5). This score is based on three main features of study quality including randomisation, blinding and description

of dropout or withdrawals. Possible scores ranged from 0 to 5 and a score of ≤ 2 indicates high risk while a score of ≥ 3 indicates low risk of bias (Jadad *et al.*, 1996).

Publication bias was evaluated by visual inspection of the funnel plot and by Egger's regression test (Egger *et al.*, 1997). 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).

2.3 Results

2.3.1 ***Search results***

The process followed in the selection of eligible studies is summarized in Figure 2.1. After full text examination, 18 RCTs were included in the systematic review.

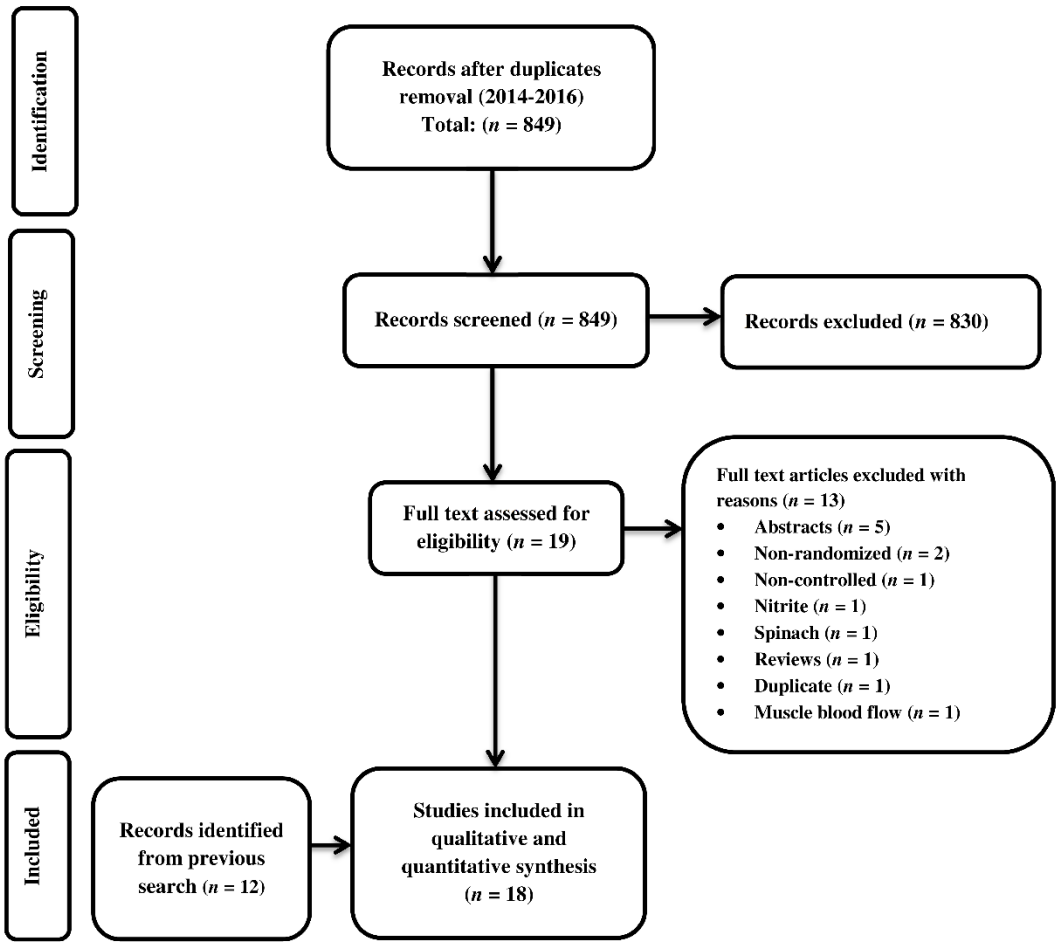


Figure 2.1: Flow diagram of the process used in selection of the studies reporting randomised controlled trials included in this systematic review and meta-analysis

2.3.2 *Studies characteristics*

Characteristics of the studies included in the systematic review and meta-analysis are summarized in Table 2.1. The total number of participants was 383 with a median sample size of 15 participants per study (range 8 – 67). The studies included 217 males and 139 females, but two studies did not report the gender of their participants (Rossi *et al.*, 2004; Bahra *et al.*, 2012). Participant age ranged from 22 to 67.2 years (median 42 years). The dose of inorganic NO_3^- ranged from 1.1 to 22.5 mmol/day and the median dose was 7.75 mmol/day. The duration of the studies ranged from 1-42 days. The study designs comprised 14 crossover (Panza *et al.*, 1993; Arcaro *et al.*, 1999; Newton *et al.*, 2001; Tounian *et al.*, 2001; Ceriello *et al.*, 2002; Rossi *et al.*, 2004; Bitar *et al.*, 2005; Correia and Haynes, 2007; Webb *et al.*, 2008; Shirwany and Zou, 2010; Bahra *et al.*, 2012; Roustit and Cracowski, 2013; Higashi, 2015; Lee *et al.*, 2015) and 4 parallel group studies (Porter, 1993; Rammos *et al.*, 2014; van Sloten *et al.*, 2014; Velmurugan *et al.*, 2016).

Table 2.1: Characteristics of studies included in the systematic review and meta-analysis

AIx: augmentation index, DB: double-blind, CO: crossover, FBF: forearm blood flow, FMD: flow mediated dilation, LDF: laser Doppler flow, P: parallel, PWV: pulse wave velocity, UB: non-blinded,

Author	Study Design	Health Status	Outcomes	Age (years)	Sample Size (Male)	NO_3^- Dose (mmol)	Type	Duration (days)	Baseline BMI (kg/m^2)	Baseline systolic BP (mmHg)	Baseline diastolic BP (mmHg)	NO_3^- Concentration before (µM/L)	NO_3^- concentration after (µM/L)	Washout Period	Jadad's Score
Porter (1993)	UB, P	Healthy	LDF	62.7	21 (12)	5.6	Beetroot	21	30.5	135.1	77.5				3
Bahra *et al.* (2012)	DB, CO	Healthy	FMD, PWV	27.9	14 (NR)	8	Inorganic NO_3^-	1	24.5	116.9	67.7	31.1	216	1-4 weeks	3
Panza *et al.* (1993)	DB, CO	Healthy	FMD	25	11 (7)	5	Beetroot	1	22	115	70			24 hours	4
Rossi *et al.* (2004)	UB, CO	Hypertension	PWV	52.9	15 (NR)	3.5	Beetroot	1	26.2	139.9	86.5	46.8	156.8		2
Tounian *et al.* (2001)	DB, CO	Type 2 diabetes	LDF, FMD	67.2	27 (18)	7.5	Beetroot	14	30.8	142.9	81.1	31	150	4 weeks	3
Arcaro *et al.* (1999)	DB, CO	Healthy	FMD	26	10 (5)	11.3	Inorganic NO_3^-	1				13	261		4
Ceriello *et al.* (2002)	UB, CO	Healthy	LDF, PWV, Aix	31	23 (23)	1.1	Beetroot	1	23.3	124	74	8.6	30.2	24 hours	2
Bitar *et al.* (2005)	UB, CO	Healthy	FMD, PWV	61	20 (20)	8.1	Beetroot	1	30.1	135.2	93.2			1 week	2
van Sloten *et al.* (2014)	DB, P	Hypertension	FMD, PWV, Aix	57.6	64 (26)	6.4	Beetroot	28	26.8	149	88.9				3
Higashi (2015)	DB, CO	Healthy	FMD	24.7	12 (5)	5.5	Beetroot	1		104	63	34		1 week	2
Newton *et al.* (2001)	UB, CO	Peripheral arterial Disease	FMD	67	8 (4)	18.2	Beetroot	1	28.6	140	76	75	475	1-2 weeks	2
Roustit and Cracowski (2013)	DB, CO	Healthy	FMD, PWV	22	12 (12)	12.9	Beetroot	1	25	120	64	31	561	5 days	5
Lee *et al.* (2015)	DB, CO	Healthy	FMD	22	14 (14)	6.4	Beetroot	15	23.4	116	77	83.8	167.6	2 weeks	4
Rammos *et al.* (2014)	DB, P	Hypertension	FMD, PWV, Aix	63.7	20 (13)	11.3	Inorganic NO_3^-	28	24	137	80	32	263		3
Shirwany and Zou (2010)	DB, CO	Healthy	FMD	25	15 (15)	12.1	Inorganic NO_3^-	1	24.1	119	68		238	1 week	4
Velmurugan *et al.* (2016)	DB, P	Hypercholesterolemia	FMD, PWV, Aix	53.3	67 (24)	6	Beetroot	42	26.8	125.2	76.3	25	200		5
Webb *et al.* (2008)	UB, CO	Healthy	FMD	26.6	10 (4)	22.5	Beetroot	1	21.3			25	400	1 week	2
Correia and Haynes (2007)	DB, CO	Heart Failure	FBF, Aix	65.5	20 (15)	12.9	Beetroot	1	35.4			10	326	10 days	3

Different methods were used to assess changes in EF in the trials. The most commonly used methods were FMD (14 trials), PWV (8 trials) and AIx (6 trials). Some trials used LDF (3 trials) and only one study used FBF (Table 2.1). Eleven studies investigated the effect of inorganic NO_3^- or beetroot in healthy volunteers (Panza *et al.*, 1993; Porter, 1993; Arcaro *et al.*, 1999; Ceriello *et al.*, 2002; Bitar *et al.*, 2005; Webb *et al.*, 2008; Shirwany and Zou, 2010; Bahra *et al.*, 2012; Roustit and Cracowski, 2013; Higashi, 2015; Lee *et al.*, 2015). The remaining studies were conducted in participants with cardiovascular and metabolic conditions including hypertension (Rossi *et al.*, 2004; Rammos *et al.*, 2014; van Sloten *et al.*, 2014), heart failure (Correia and Haynes, 2007), peripheral arterial disease (Newton *et al.*, 2001), hypercholesterolemia (Velmurugan *et al.*, 2016) and type 2 diabetes (Tounian *et al.*, 2001).

2.3.3 ***Qualitative analysis***

Two thirds of the studies (12 studies) included in this meta-analysis were of moderate-high quality on the Jadad score (≥ 3) while the rest of studies (6 studies) appeared to have a high risk of bias (Table 2.1). Individually, 13 studies reported that inorganic NO_3^- or beetroot supplementation improved endothelial function significantly. Six studies reported adverse effects including beeturia or stool discoloration (Newton *et al.*, 2001; Tounian *et al.*, 2001; Ceriello *et al.*, 2002; Zieman *et al.*, 2005; van Sloten *et al.*, 2014; Velmurugan *et al.*, 2016). Twelve studies reported the dropout rate during the interventions (Panza *et al.*, 1993; Porter, 1993; Tounian *et al.*, 2001; Ceriello *et al.*, 2002; Rossi *et al.*, 2004; Bitar *et al.*, 2005; Correia and Haynes, 2007; Webb *et al.*, 2008; Shirwany and Zou, 2010; van Sloten *et al.*, 2014; Higashi, 2015; Velmurugan *et al.*, 2016). The washout period used in the crossover trials varied from 1 day to 4 weeks and two studies failed to report this information (Arcaro *et al.*, 1999; Rossi *et al.*, 2004).

2.3.4 *The effects of inorganic NO_3^- or beetroot on FMD*

Data synthesis revealed significant increase in FMD with inorganic NO_3^- or beetroot interventions (WMD: 1.36%, 95% CI: 0.73, 1.99, $P < 0.001$). However, these studies were characterized by significant heterogeneity ($X^2 = 76.8$, $P < 0.001$, $I^2 = 83.1\%$) (Figure 2.2). Subgroup analyses (Table 2.2) demonstrated a significantly greater effect in healthy participants in comparison with those with underlying diseases (WMD: 2.25%, 95% CI: 1.35, 3.14, $P < 0.001$). Likewise, inorganic NO_3^- or beetroot showed higher efficacy in non-obese participants (BMI < 25 kg/m2) (WMD: 1.49%, 95% CI: 0.74, 2.23, $P < 0.001$).

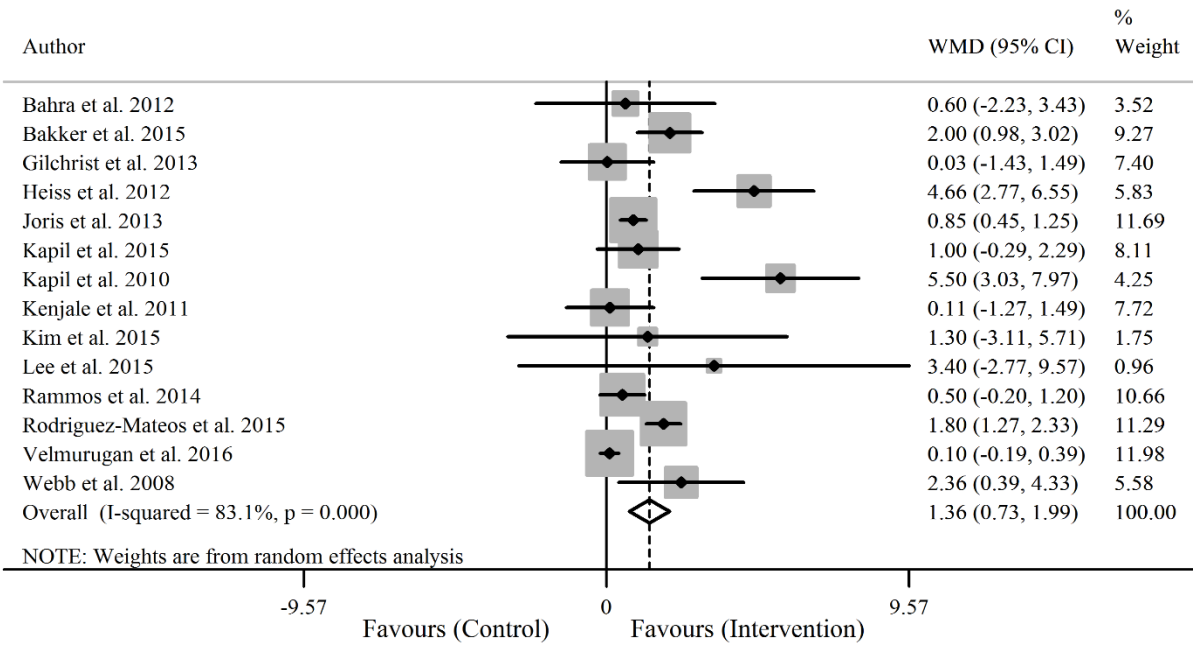


Figure 2.2: Forest plot showing the effect of inorganic NO_3^- or beetroot on flow mediated dilation. Diamonds indicate the effect size of each study summarized as weighted mean difference (WMD). The size of the shaded squares is proportional to the percentage weight of each study. Horizontal lines represent the 95% confidence interval and the vertical broken line represents the overall effect.

Table 2.2: Subgroup analysis of the effect of inorganic NO_3^- or beetroot on flow mediated dilation

	No. of subgroups	Effect size	95% CI	*P* Value	*P* between subgroups	I^2 (%)
Health status					0.01	
Healthy	9	2.25	1.35 to 3.14	0.001		76.6
Disease	5	0.18	-0.07 to 0.44	0.152		0
Age of participants (years)					0.07	
< 60 years	10	2.06	1.00 to 3.12	0.001		87.9
≥ 60 years	4	0.69	0.36 to 1.02	0.001		0
Body mass index (kg/m^2)					0.04	
< 25	6	1.49	0.74 to 2.23	0.001		56.0
≥ 25	6	0.45	-0.02 to 0.92	0.06		51.2
Systolic blood pressure (mmHg)					0.14	
< 130	7	1.79	0.55 to 3.04	0.005		88.9
≥ 130	5	0.71	0.39 to 1.03	0.001		0
Diastolic blood pressure (mmHg)					0.81	
< 85	10	1.15	0.33 to 1.97	0.006		83.8
≥ 85	2	0.86	0.48 to 1.25	0.001		0
Type of intervention					0.59	
Beetroot	10	1.13	0.44 to 1.82	0.001		77.5
Inorganic NO_3^-	4	1.82	0.44 to 3.19	0.009		85.1
Study Design					0.16	
Parallel	3	0.27	-0.12 to 0.66	0.172		24.5
Crossover	11	1.77	0.98 to 2.56	0.001		75.5
Study Quality (Jadad Score)					0.61	
Low risk (4-6)	10	1.28	0.46 to 2.09	0.002		84.5
High risk (2-3)	4	1.86	0.24 to 3.48	0.025		82.0

Meta-regression analyses (Figure 2.3) revealed significant reduction in the effects of inorganic NO_3^- or beetroot on FMD in older participants (β: -0.05, 95% CI: -0.09, 0.02, $P < 0.01$) and in those with higher baseline systolic BP (β: -0.06, 95% CI: -0.12, 0.01, $P = 0.02$).

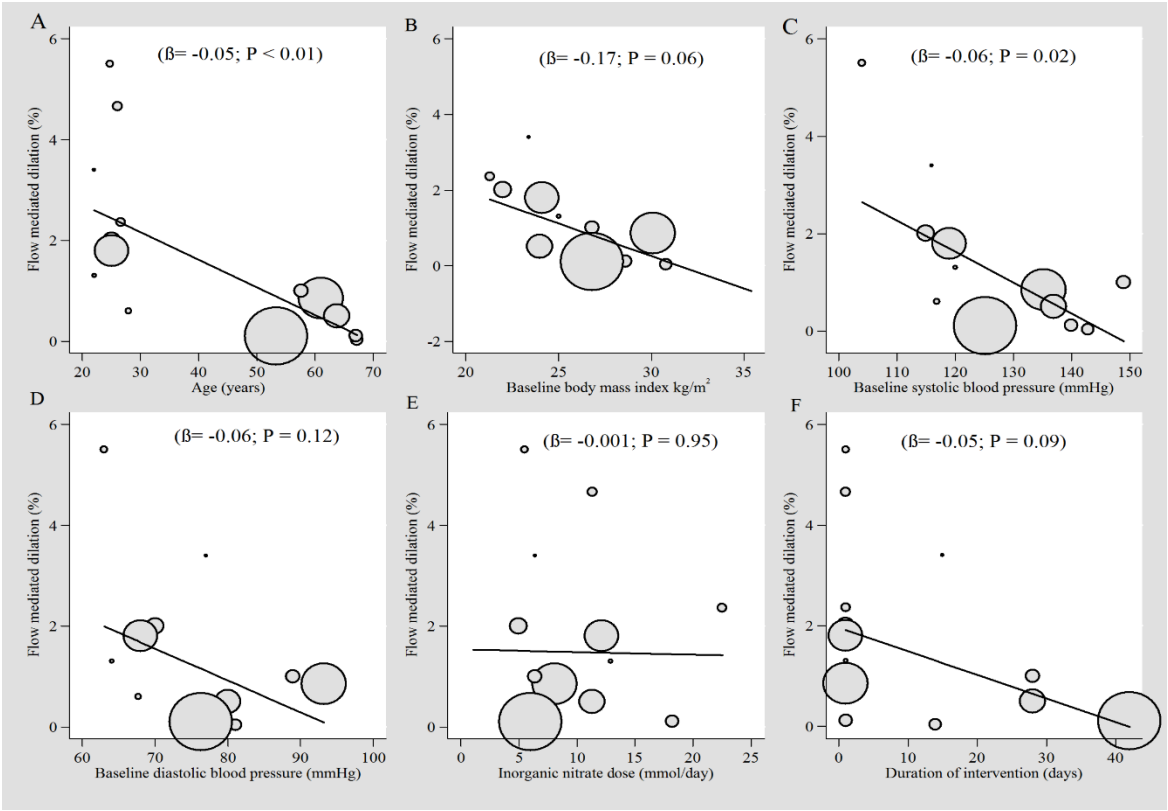


Figure 2.3: Associations between inorganic NO_3^- or beetroot supplementation effects on flow mediated dilation and participants' characteristics (age, baseline body mass index, systolic or diastolic blood pressure) or of studies characteristics (dose and duration of NO_3^- intervention). Each study is depicted by a circle where the circle size represents the degree of weighting for the study based on participant numbers in the study.

2.3.5 *The effects of inorganic NO_3^- or beetroot on PWV*

Inorganic NO_3^- or beetroot supplementation had no detectable effect on PWV (WMD: -0.05 m/s, 95% CI: -0.38, 0.28, P = 0.75) (Figure 2.4). There was no significant heterogeneity between trials (X^2 = 11.3, P = 0.13, I^2 = 38.2). Subgroup analyses of studies showed no significant differences between groups based on participant or study characteristics (Table 2.3). However, meta-regression analyses (Figure 2.5) demonstrated marginally significant relationship between the changes in PWV and the dose of inorganic NO_3^- (β: -0.09, 95% CI: -0.18, -0.002, P = 0.05).

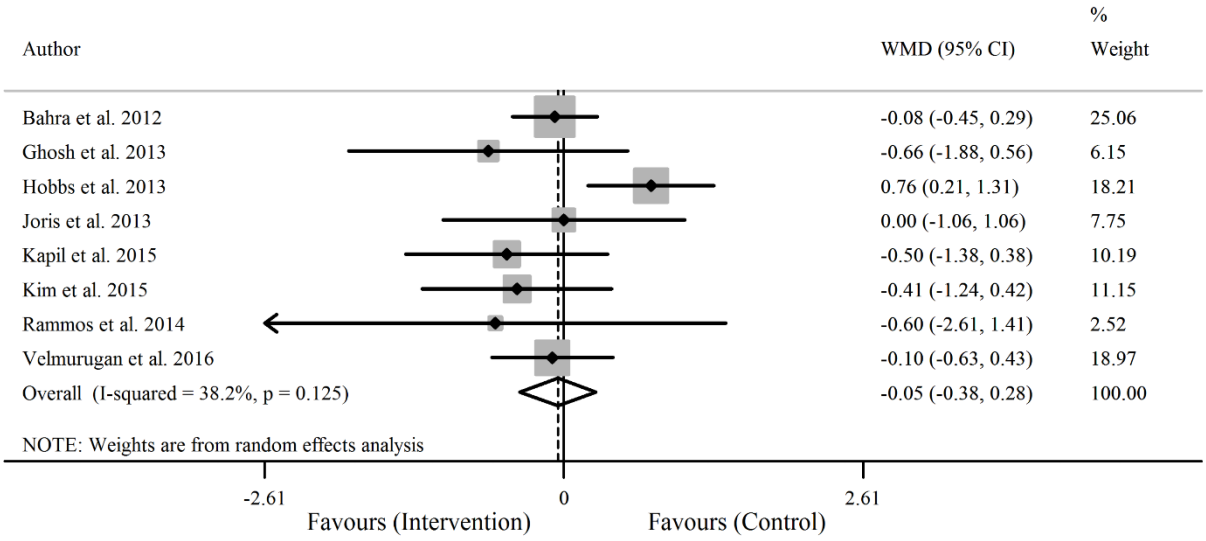


Figure 2.4: Forest plot showing the effect of inorganic NO_3^- or beetroot on pulse wave velocity. Diamonds indicate the effect size of each study summarized as weighted mean difference (WMD). The size of the shaded squares is proportional to the percentage weight of each study. Horizontal lines represent the 95% confidence interval and the vertical broken line represents the overall effect.

Table 2.3: Subgroup analysis of the effect of inorganic NO_3^- and beetroot on pulse wave velocity

	No. of subgroups	Effect size	95% CI	*P* Value	*P* between subgroups	I^2 (%)
Health status					0.26	
Healthy	4	0.11	-0.41 to 0.63	0.686		62.3
Disease	4	-0.28	-0.69 to 0.14	0.194		0
Age of participants (years)					0.87	
< 60 years	6	-0.06	-0.45 to 0.33	0.774		54.5
≥ 60 years	2	-0.13	-1.06 to 0.81	0.786		0
Body mass index (kg/m^2)					0.18	
< 25	3	0.22	-0.51 to 0.94	0.561		70.4
≥ 25	5	-0.26	-0.61 to 0.09	0.153		0
Systolic blood pressure (mmHg)					0.27	
< 130	4	0.07	-0.38 to 0.52	0.761		64.2
≥ 130	4	-0.40	-0.97 to 0.17	0.168		0
Diastolic blood pressure (mmHg)					0.33	
< 85	5	0.04	-0.38 to 0.47	0.837		54.5
≥ 85	3	-0.38	-0.97 to 0.21	0.206		0
Type of intervention					0.85	
Beetroot	6	-0.06	-0.53 to 0.41	0.798		53.4
Inorganic NO_3^-	2	-0.10	-0.46 to 0.27	0.602		0
Study Design					0.46	
Parallel	3	-0.22	-0.67 to 0.22	0.319		0
Crossover	5	0.02	-0.47 to 0.51	0.932		57.4
Study Quality (Jadad Score)					0.08	
Low risk (4-6)	5	-0.17	-0.44 to 0.10	0.219		0
High risk (2-3)	3	0.17	-0.68 to 1.02	0.695		60.8

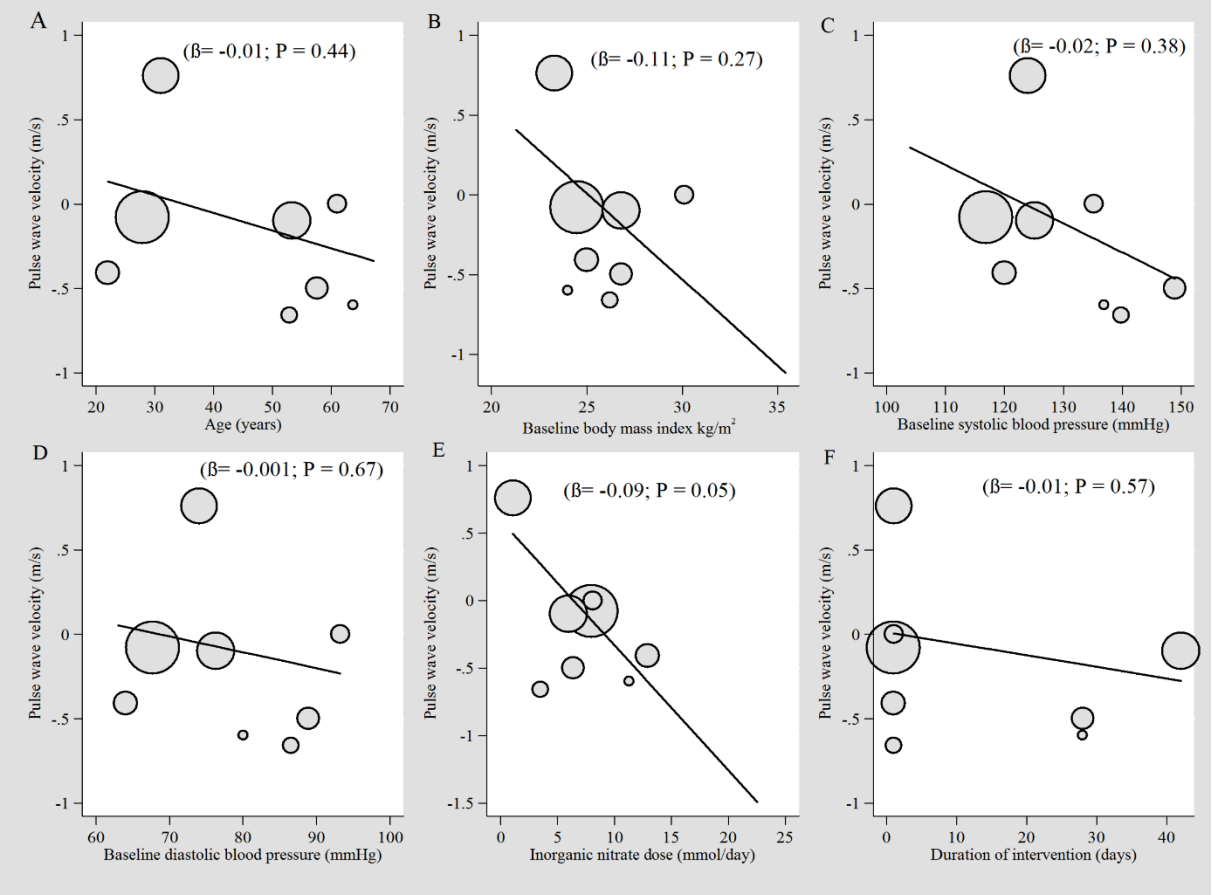


Figure 2.5: Associations between inorganic NO_3^- or beetroot supplementations effects on pulse wave velocity and characteristics of participants (age, baseline body mass index, systolic or diastolic blood pressure) or of studies characteristics (dose and duration of NO_3^- intervention). Each study is depicted by a circle where the circle size represents the degree of weighting for the study based on participant numbers in the study.

2.3.6 *The effects of inorganic NO_3^- or beetroot on AIx*

Meta-analysis revealed no effect of inorganic NO_3^- or beetroot supplementation on AIx (WMD: -1.73 %, 95% CI: -3.85, 0.40, P = 0.11) (Figure 2.6). There was no significant heterogeneity between trials (X^2 = 2.65, P = 0.73, I^2 = 0). Because of the small number of studies (6 trials), we could not conduct subgroup analyses. However, meta-regression analyses found no evidence that characteristics of participants or of studies influenced the effect of inorganic NO_3^- or beetroot on AIx (Figure 2.7).

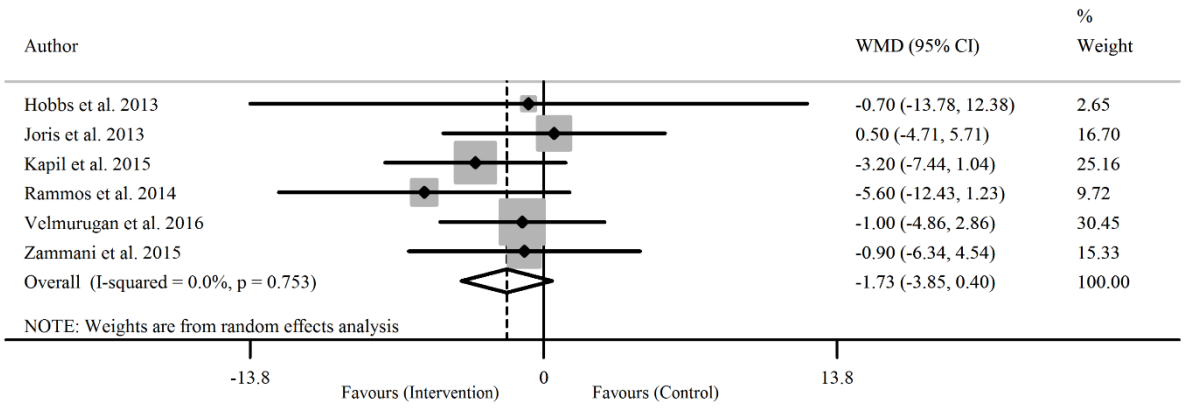


Figure 2.6: Forest plot showing the effect of inorganic NO_3^- or beetroot on augmentation index (%). Diamonds indicate the effect size of each study summarized as weighted mean difference (WMD). The size of the shaded squares is proportional to the percentage weight of each study. Horizontal lines represent the 95% confidence interval and the vertical broken line represents the overall effect.

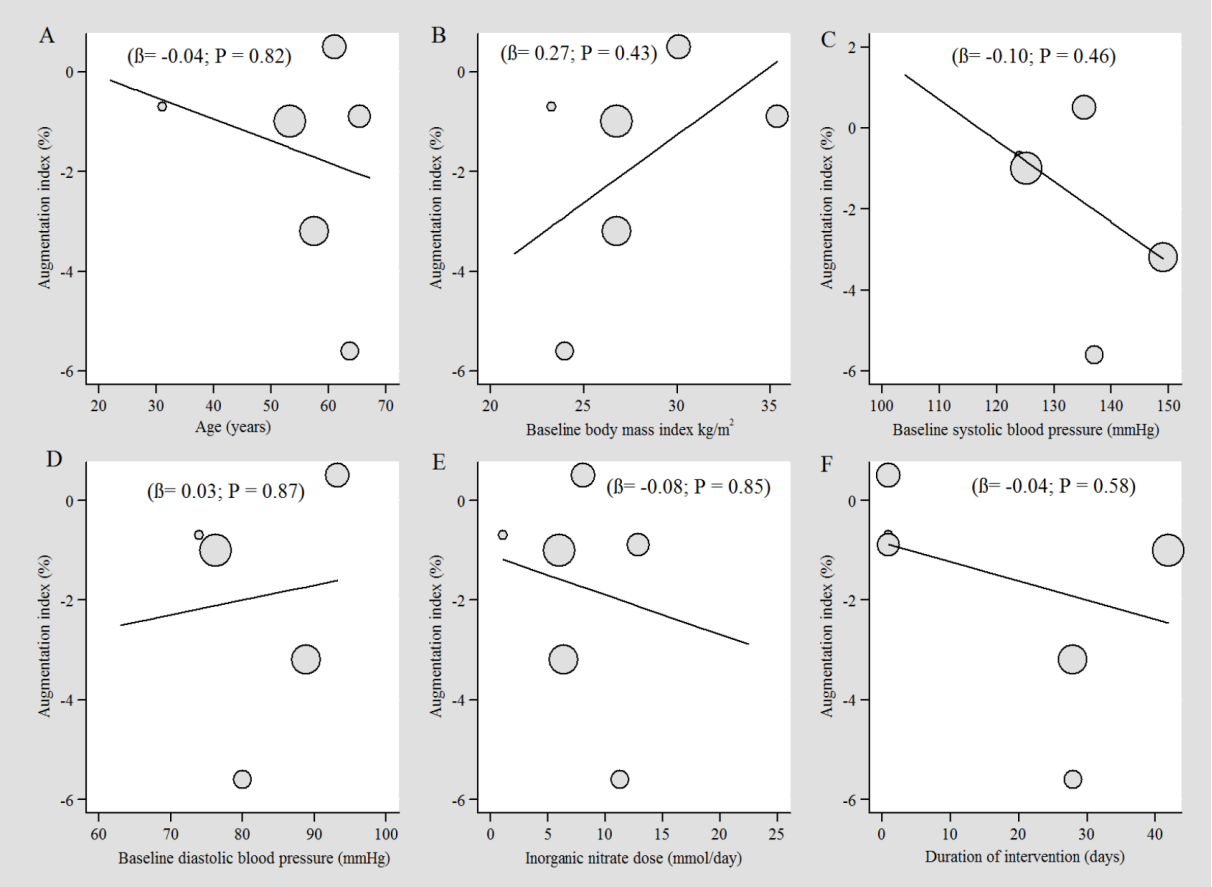


Figure 2.7: Associations between inorganic NO_3^- or beetroot supplementations effects on augmentation index and characteristics of participants (age, baseline body mass index, systolic or diastolic blood pressure) or of studies characteristics (dose and duration of NO_3^- intervention). Each study is depicted by a circle where the circle size represents the degree of weighting for the study based on participant numbers in the study.

2.3.7 ***Publication bias***

The funnel plots (Figure 2.8) showed asymmetry in the distribution of the studies included in the three meta-analyses (FMD, PWV and AIx) which may denote either publication bias or small study effect (the tendency of small studies to show higher effect sizes than larger studies). However, Egger's regression test (β: 0.20, $P = 0.05$; β: 0. 28, $P = 0.37$; β: -1.14, $P = 0.84$ for FMD, PWV and AIx, respectively) suggest low likelihood of publication bias.

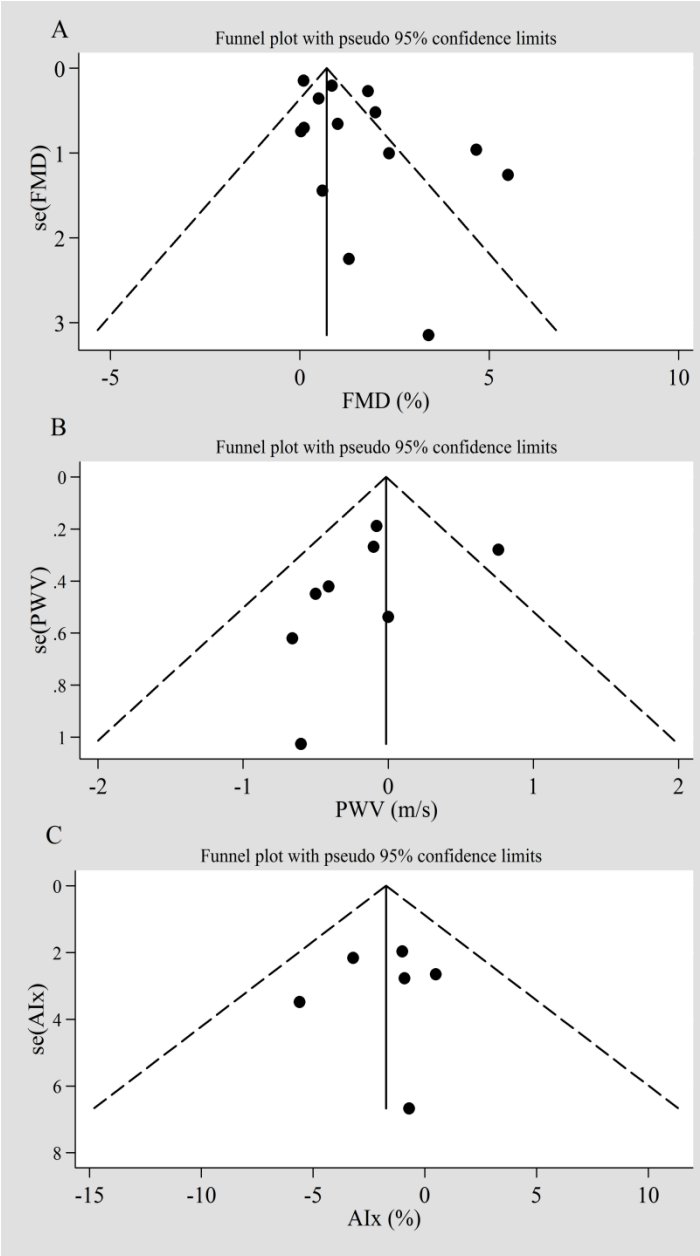


Figure 2.8: Funnel plot for publication bias of the effect of inorganic NO_3^- or beetroot supplementations on, A) flow mediated dilation (FMD); B) pulse wave velocity (PWV); C) augmentation index (Aix).

2.4 Discussion

Inorganic NO_3^- and beetroot supplementations were associated with a significant improvement in FMD. However, neither PWV nor AIx was improved with these interventions. Nevertheless, inorganic NO_3^- or beetroot administration in larger doses might be associated with greater reduction in PWV. The improvement in EF tends to be smaller in older, overweight/obese participants and in those with underlying cardio-metabolic disease.

The biological mechanisms explaining the apparent influence of ageing in modifying the effect of inorganic NO_3^- on EF remain uncertain. It is possible that ageing interferes with the bio-conversion of dietary NO_3^- to nitric oxide e.g. by modification of oral micro-environment which may influence the efficiency of bacterial reductase activity in the conversion of NO_3^- into NO_2^- (Percival *et al.*, 1991). In addition, gastric acid production declines with age (Britton and McLaughlin, 2013) and this process may affect the formation of NO in the acid stomach from the acid-mediated disproportionation of NO_2^- (Zweier *et al.*, 1999). Disproportionation is a specific type of redox reaction in which an element from a reaction undergoes both oxidation and reduction to form two different products. Epidemiological studies showed a 4.8% prevalence of atrophic gastritis (reduction in the number of acid-producing cells) in population aged 50-54 years; this number bounced to 8.9% in those aged > 70 years (Brownie, 2006; Weck *et al.*, 2007). In the current study, we found around 70% reduction in the effect of NO_3^- on FMD in > 60 years old population (Table 2.2).

Hence, it is currently not known whether greater doses of inorganic NO_3^- are required in older people to account for the decline in redox potential and augment NO bioavailability. Ageing may also be associated with diminished sensitivity to the dilatory effects of NO, thus higher doses may be required (Lyons *et al.*, 1997; Montero *et al.*, 2015). In chapter four and five of this thesis, we will try to answer the question regarding the difference in absorptive capacity and efficacy of inorganic NO_3^- supplementation in two different age groups.

Whilst the main finding from this analysis is that dietary NO_3^- improves FMD, it is important to also appreciate that the duration of the interventions was relatively short (longest duration was 42 days). Furthermore, the meta-regression analysis showed a tendency of reduction in the effect of NO_3^- supplementation on FMD over time (Figure 2.3). Therefore, it is not known from the present meta-analysis whether the observed improvements in vascular function can be maintained in a relatively longer duration studies. This is a critical factor which needs to be evaluated to understand if NO_3^-/ beetroot interventions have the potential to be effective

strategies for the primary and/or secondary prevention of endothelial dysfunction and atherosclerosis.

The pathological process behind the deterioration of PWV and AIx reflect three potential risk factors including systolic BP, pulse pressure and structural changes in the arterial wall (Jani and Rajkumar, 2006). Therefore, any interventions expected to improve PWV and AIx need to improve these risk factors. In contrast with the functional alteration associated with endothelial dysfunction (measured by FMD) (Seals *et al.*, 2011), the structural alteration in the arterial wall needed a longer duration intervention to observe a clinically reasonable effect. The current study revealed a non-significant effect of inorganic NO_3^- on PWV and AIx. This may be linked to the short-term duration of the studies which is unlikely to induce structural changes of the arterial wall and have an effect on pulse wave transit and reflection.

This study has several potential limitations. One limitation of the present analysis is the high levels of heterogeneity among the FMD studies and hence our findings should be treated with caution. The considerable variability in the design, duration, dose of dietary NO_3^- and participant characteristics (age, sex, health status) may have contributed to the significant heterogeneity observed in our meta-analysis. We undertook subgroup analysis to explore a number of potential sources of heterogeneity; but, heterogeneity remained at moderate to high levels. The asymmetry observed in the funnel plot may be evidence of publication bias. However, the latter observation should be interpreted cautiously. Apparent asymmetry in such funnel plots may be due to factors other than the presence of publication bias including: 1) most of the included studies had small sample size (small study effect). The small study effect means that small trials tend to report greater treatment effect than large trials and therefore causes asymmetry of the funnel plot (Nuesch *et al.*, 2010); 2) heterogeneity in design and in outcome measures between studies (there is substantial clinical and methodological heterogeneity among the studies included in our meta-analysis), and studies involving different populations (Lau *et al.*, 2006).

The meta-analyses are based on retrospective analytical inference which may be affected by several factors such as inclusiveness of the search strategy to identify eligible studies, assumptions on consistency of methodologies applied across the different studies, inconsistency in reporting study results and limited accessibility to individual study data. In addition, we have limited our search to articles published in English. The clear delineation of a priori inclusion and exclusion criteria and the comprehensive search of three major electronic databases and

reference lists are likely to have minimized bias and increased the representativeness of the results.

2.4.1 *Conclusions*

This systematic review and meta-analysis revealed a significant improvement of EF after inorganic NO_3^- and beetroot juice supplementation. If such improvements were sustained in the long-term, these finds have potential implications for the prevention of atherosclerosis and cardiovascular diseases. However, before the results can be translated into new nutritional recommendations, the long-term effects of inorganic NO_3^- on endothelial function need to be investigated in older subjects and in patients at higher cardiovascular risk. Importantly, the present findings suggest that inorganic NO_3^- supplementation may be less effective, at least in the short-term, in such population groups.

Chapter 3. Vitamin C and biomarkers of cardiovascular diseases: An umbrella review

3.1 Introduction

Vitamin C is an essential nutrient which has antioxidant roles in the human body (Padayatty *et al.*, 2003). It is regarded by some investigators as the most important antioxidant in human plasma (Halliwell, 1996). Ascorbic acid (the reduced form of vitamin C) scavenges physiologically relevant reactive oxygen and nitrogen species (Carr and Frei, 1999). Similarly, vitamin C can reduce non-radical species and can regenerate circulatory antioxidant molecules (Figure 3.1) (Carr and Frei, 1999).

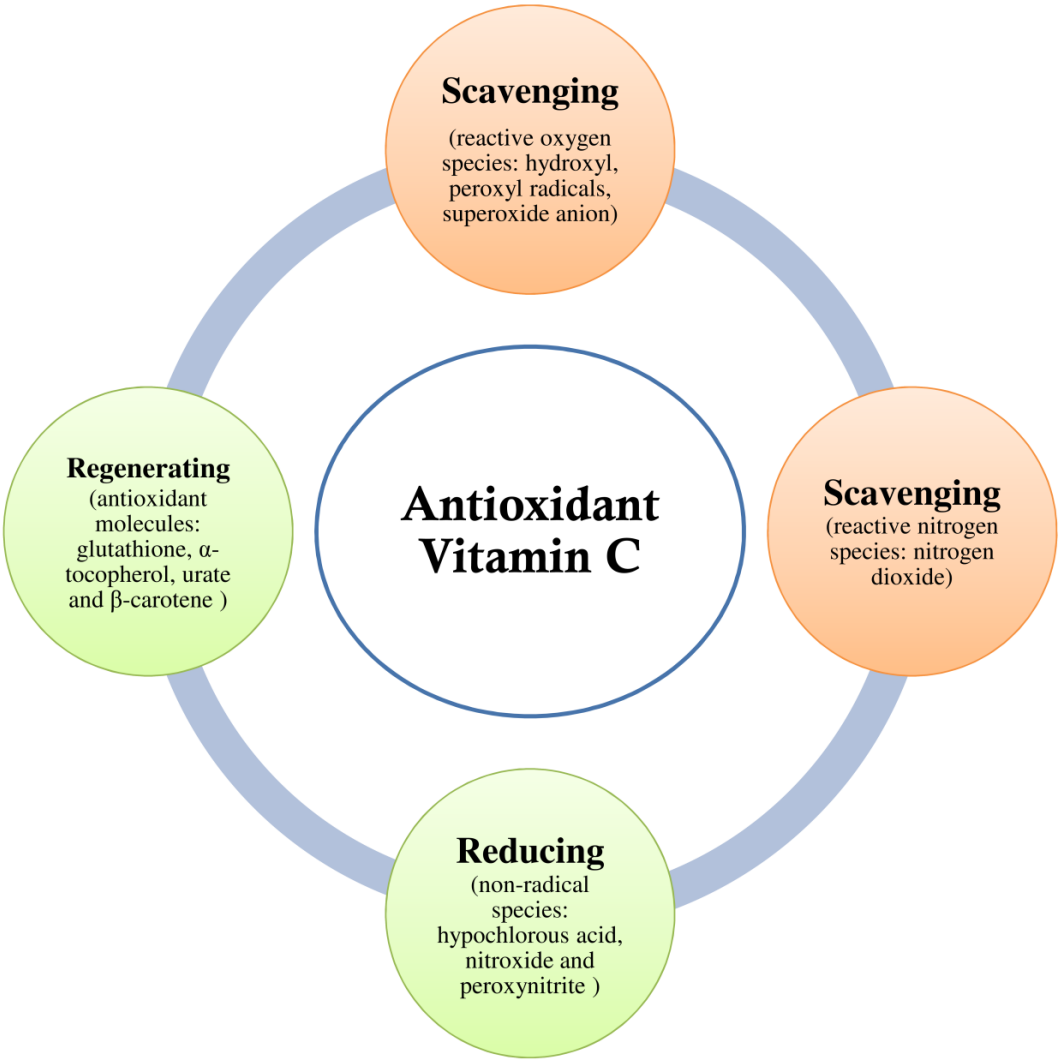


Figure 3.1: Antioxidant functions of vitamin C

Regardless of the extensive evidence of antioxidant action, studies have shown that vitamin C can also behave as a prooxidant in certain laboratory settings (e.g. high oxygen tension and in the presence of metal ions) (Padayatty and Levine, 2016). Vitamin C has been considered as a perplexing micronutrient *"Of all the paradoxical compounds, ascorbic acid probably tops the list. It is truly a two-headed Janus, a Dr. Jekyll-Mr. Hyde, an oxymoron of antioxidants"* (Porter, 1993).

Antioxidants such as vitamin C proved to be beneficial on CVD outcomes in small experimental and epidemiological cohort studies (Li and Schellhorn, 2007). Conversely, large RCTs did not establish any superior effects of supplemental vitamin C to placebos on CVD outcomes (Honarbakhsh and Schachter, 2009). However, there are some limitations in the design and conduct of these large RCTs which confounded the interpretation of the beneficial/deleterious effects of supplementary vitamin C (Lykkesfeldt and Poulsen, 2010; Frei *et al.*, 2012; Michels and Frei, 2013). The majority of big trials used combined antioxidant vitamins so that it is impossible to separate the effects (or lack of effects) of vitamin C from the effects of the other vitamins with which it was administered (Lykkesfeldt and Poulsen, 2010). Additionally, these studies recruited well-nourished control and intervention groups (Padayatty and Levine, 2016). Pharmacokinetics studies have shown that supplementing vitamin C to well-nourished individuals will modestly increase plasma and tissue vitamin C concentrations (Levine *et al.*, 1996; Levine *et al.*, 2001). Failure of these studies to include measures of cardiovascular biomarkers (e.g. BP, arterial stiffness, EF or lipid profile) throughout the study period and correlating them to the therapeutic interventions is considered by some investigators as a major drawback in these large RCTs (Pashkow, 2011). Therefore, in this overview of systematic reviews and meta-analyses "umbrella review", I aimed to investigate the effects of supplementation of vitamin C alone on biomarkers of cardiovascular diseases. In addition, I aimed to evaluate the strength of evidence and the methodological qualities of these studies.

3.2 Methods

This umbrella review was conducted and reported according to the guidelines described by Aromataris *et al.* (2015).

3.2.1 *Data sources and search strategy*

The search for relevant systematic reviews and meta-analyses was conducted via electronic search of three databases (Medline, Embase and Scopus). Additionally, I searched for eligible studies in the reference lists of the relevant articles and reviews. The following keywords were

used to search the above databases from inception until November 2016: antioxidants, ascorbic acid, vitamin C, cardio-metabolic, cardiovascular diseases, metabolic diseases, systematic review and meta-analysis.

3.2.2 *Study selection*

The following criteria were applied to identify the articles to be included in this umbrella review: 1) systematic reviews and meta-analyses of RCTs; 2) studies involving adults aged 18 years or more and no exclusion criteria were applied for health status; 3) vitamin C administered alone i.e. not combined with other drugs or nutritional interventions; 4) studies reporting changes in CVD biomarkers (i.e. arterial stiffness, blood pressure, endothelial function, glycaemic index and lipid profile). Two investigators screened the titles and abstracts of the articles independently 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 article was moved to the full-text stage. The full-texts of the selected articles were critically appraised to determine eligibility for inclusion in the umbrella review. Disagreements were resolved by discussion between the reviewers until consensus was reached.

3.2.3 *Data extraction*

The following information was extracted from the eligible articles: 1) authors, journal details and year of publication; 2) descriptive information: databases searched, number of trials included, studies outcomes, total number of participants, age range; 3) data synthesis results: effect size, heterogeneity, subgroup analysis, meta-regression analysis and publication bias.

3.2.4 *Quality assessment*

Study quality were assessed by the modified version of the Overview of Quality Assessment Questionnaire (OQAQ) (Oxman and Guyatt, 1991). As used for assessment of systematic reviews, this tool included 9 questions: 1) were the search methods used to find evidence on the primary question(s) stated? 2) was the search for evidence reasonably comprehensive? 3) were the criteria used for deciding which studies to include in the review reported? 4) was bias in the selection of articles avoided? 5) were the criteria used for assessing the methodological quality of studies reviewed reported?; 6) were study quality assessment criteria used to inform the review analysis? 7) were the methods used to combine the findings of the relevant studies (to reach a conclusion) reported? 8) were findings of the relevant studies combined appropriately relative to the primary question of the overview? 9) were the conclusions made by the author(s)

supported by the data and/or analysis reported in the overview? These questions were answered as “yes”, “no” or “partially/can’t tell” and then the answers were scored 2, 0 and 1, respectively (the total score ranged from 0 to 18).

3.3 Results

3.3.1 *Search results and studies characteristics*

The process followed in the selection of eligible studies is summarized in Figure 3.2. After full text examination, 10 systematic reviews and meta-analyses were included in the umbrella review. Three studies investigated the effects of vitamin C on endothelial function (Ashor *et al.*, 2014a; Montero *et al.*, 2014; Ashor *et al.*, 2015b), two studies lipid profile (McRae, 2008; Ashor *et al.*, 2016c), two studies glycaemic index (Tabatabaei-Malazy *et al.*, 2014; Ashor *et al.*, 2017) and one study each for arterial stiffness (Ashor *et al.*, 2014b), blood pressure (Juraschek *et al.*, 2012) and insulin resistance (Khodaeian *et al.*, 2015) (Table 3.1).

The number of trials included in each study ranged from 3 to 44 trials, included participants ranged from 92 to 1981 participants per meta-analysis. The age of participants ranged from 20 to 82 years. Two studies did not report the number and age of the included participants (Montero *et al.*, 2014; Tabatabaei-Malazy *et al.*, 2014) (Table 3.1). The OQAQ quality score of the studies ranged from 11 to 18 (Table 3.2). Summary of the findings from these meta-analysis studies are presented in (Figure 3.3) below.

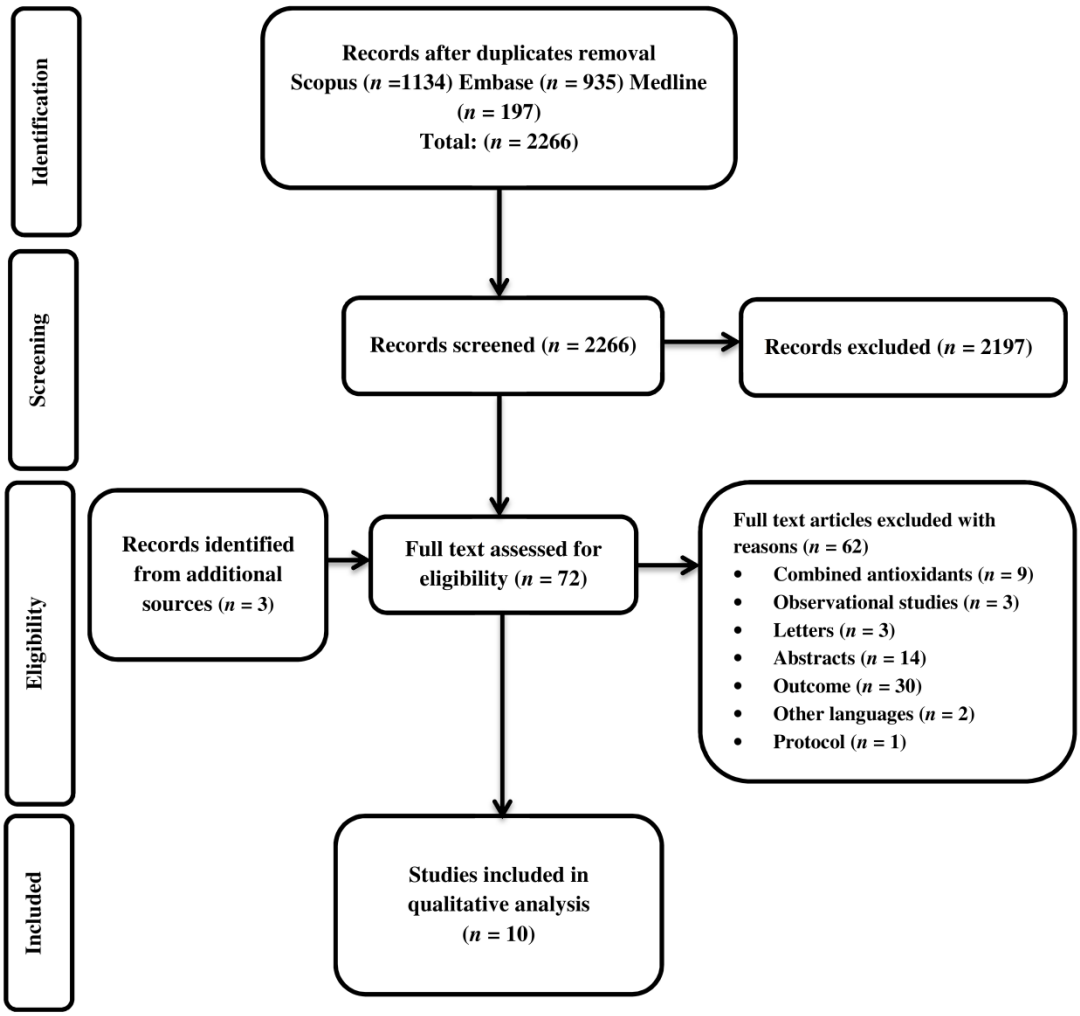


Figure 3.2: Flow diagram of the process used in selection of the systematic reviews and meta-analyses studies included in this umbrella review

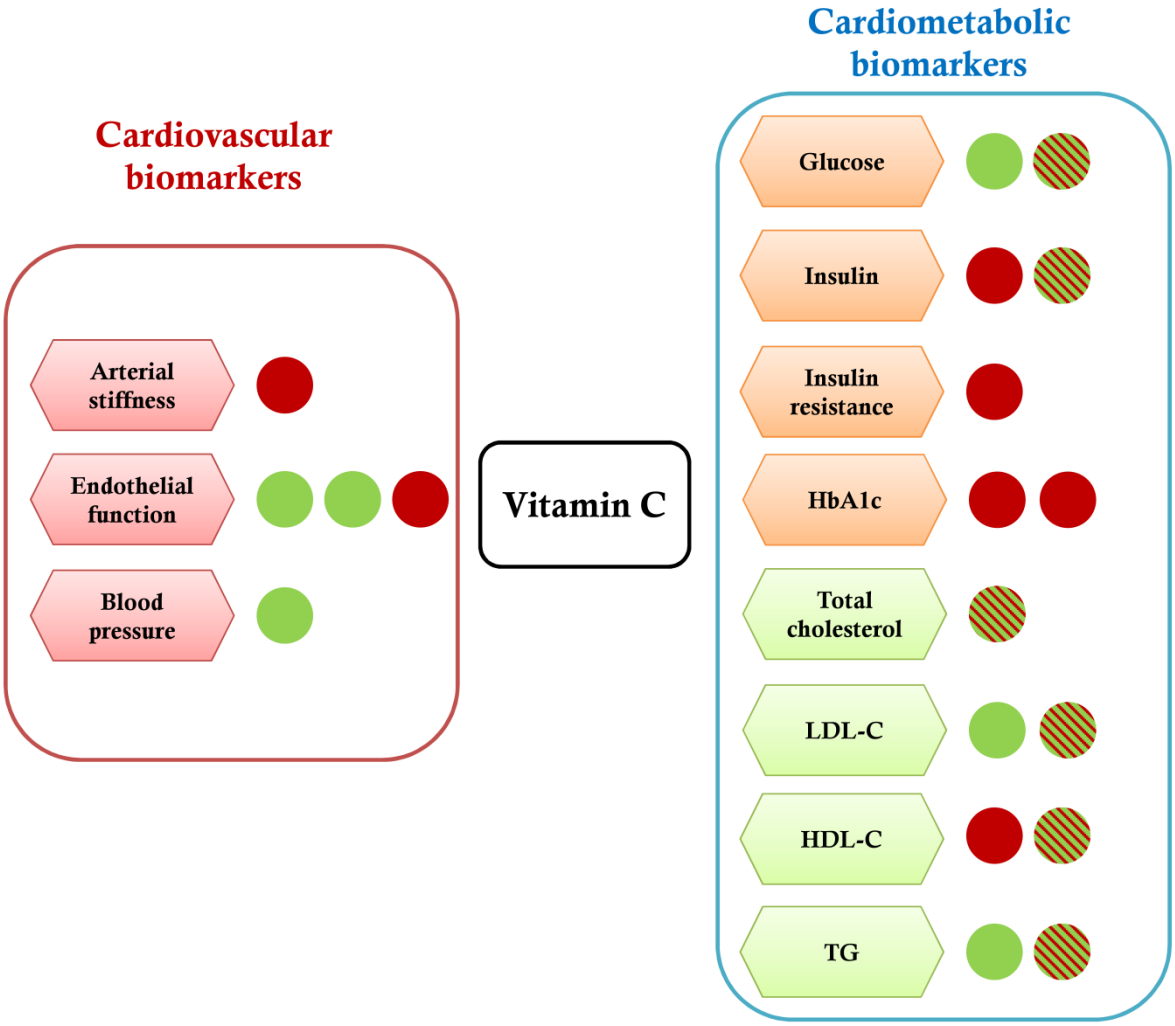


Figure 3.3: Summary of the findings from studies included in vitamin C and cardiovascular biomarkers umbrella review. Each circle represents a meta-analysis study. Green circles denote positive effect, red circles denote absence of effect and circles with green and red pattern represent absence of an overall effect, however, subgroup and meta-regression analyses demonstrated positive effects. HbA1c: haemoglobin A1C; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides

Table 3.1: Description of the 10 systematic reviews and meta-analyses of vitamin C and cardiovascular biomarkers included in this umbrella review

Study	Outcome	Database searched	Search range (years)	Number of trials	Number of participants	Age range (years)
Ashor et al. 2014	Endothelial function	PubMed, Embase, Scopus and Cochrane Library	from inception to May 2013	44	1129	22-68
Ashor et al. 2015	Endothelial function	MEDLINE, Embase, Scopus and Cochrane Library	from inception to May 2014	17	478	23-78
Ashor et al. 2014	Arterial stiffness	MEDLINE, Embase and Scopus	from inception to December 2013	10	273	22-63.5
Ashor et al. 2015	Lipid profile	PubMed, Embase, Scopus and Cochrane Library	from inception to August 2014	40	1981	20-81
Ashor et al. 2017	Glycaemic control	PubMed, Embase, Scopus, and Cochrane Library	from inception until July 2015	24	998	22 – 72
Juraschek et al. 2012	Blood pressure	MEDLINE, Embase and Cochrane Library	1966-2011	29	1407	22-74
Khodaeian et al. 2015	Insulin resistance	Google Scholar and PubMed	from inception to January 2014	3	92	39-72+
McRae 2008	Lipid profile	MEDLINE	1970-2007	13	549	48-82
Montero et al. 2013	Endothelial function	MEDLINE, Cochrane, Scopus and Web of Science	from inception to February 2013	6	NR	NR
Tabatabaei-Malazy et al. 2014	Glycaemic control	Google Scholar and PubMed, Scopus, IranMedex and Magiran web	from inception to January 2013	12	NR	NR

NR: not reported

Table 3.2: Quality assessment of the 10 systematic reviews and meta-analyses of vitamin C and cardiovascular biomarkers included in this umbrella review

Study	1. Were the search methods used to find evidence on the primary question(s) stated?	2. Was the search for evidence reasonably comprehensive?	3. Were the criteria used for deciding which studies to include in the review reported?	4. Was bias in the selection of articles avoided?	5. Were the criteria used for assessing the methodological quality of studies reviewed reported?	6. Were study quality assessment criteria used to inform the review analysis?	7. Were the methods used to combine the findings of the relevant studies (to reach a conclusion) reported?	8. Were findings of the relevant studies combined appropriately relative to the primary question of the overview?	9. Were the conclusions made by the author(s) supported by the data and/or analysis reported in the overview?	Sum quality score
Ashor et al. 2014	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	18/18
Ashor et al. 2015	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	18/18
Ashor et al. 2014	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	18/18
Ashor et al. 2015	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	18/18
Ashor et al. 2017	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	18/18
Juraschek et al. 2012	Yes	Partial	Yes	Yes	Yes	Partial	Yes	Yes	Yes	16/18
Khodaeian et al. 2015	Yes	Yes	Yes	Partial	Yes	Partial	Yes	Yes	Partial	15/18
McRae 2008	Yes	Yes	Yes	Partial	No	No	Partial	Yes	Yes	11/18
Montero et al. 2013	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	18/18
Tabatabaei-Malazy et al. 2014	Yes	Partial	Yes	Partial	Yes	No	Yes	Yes	Yes	14/18

3.3.2 ***Vitamin C and cardiovascular biomarkers***

a. Vitamin C and arterial stiffness: Data synthesis from 10 trials (273 participants aged between 22-63.5 years) revealed no significant effects of vitamin C supplementation on indices of arterial stiffness (PWV, AIx, compliance coefficient, distensibility coefficient or digital volume pulse) (SMD: -0.16; 95% CI: -0.42, 0.11) (Ashor *et al.*, 2014b). In addition, low heterogeneity between studies was reported (I^2 = 29.2%) (Table 3.3).

b. Vitamin C and blood pressure: The single systematic review and meta-analysis (29 trials, 1407 participants aged between 22-74 years) which investigated effects on BP found that vitamin C supplementation reduced both systolic (WMD: -3.84 mmHg; 95% CI: -5.29, -2.38) and diastolic BP (WMD: -1.48 mmHg; 95% CI: -2.86, -0.10) (Juraschek *et al.*, 2012). However, there was high heterogeneity between studies (systolic BP: I^2 = 69%; diastolic BP: I^2 = 81%). In subgroup analyses, the authors reported greater effects in younger people, in studies with larger sample size and in studies which reported fewer BP measurements (Table 3.3).

c. Vitamin C and EF: In their meta-analysis of six trials (number and age of participants not reported), Montero *et al.* (2014) showed no significant effect of vitamin C supplementation on indices of EF (FMD, plethysmography, post-reactive hyperaemia [PORH], capillaroscopy) (Table 3.3). However, another two meta-analyses (Ashor *et al.*, 2014a; Ashor *et al.*, 2015b) revealed that vitamin C supplementation improved EF significantly.

The first study (Ashor *et al.*, 2014a), which involved 44 trials (1129 participants aged 22-68 years), demonstrated modest, but statistically significant, improvement in EF with vitamin C supplementation (SMD: 0.50, 95% CI: 0.34, 0.66). Moreover, subgroup analyses showed bigger effects in experimentally-induced endothelial dysfunction, and in patients with atherosclerosis or diabetes. Meta-regression analysis demonstrated significant correlation between vitamin C dose and the magnitude of effect on EF (Table 3.3). The second meta-analysis (Ashor *et al.*, 2015b) excluded studies in which vitamin C was supplemented for less than 2 weeks. Data synthesis of 17 trials (478 participants with age range of 23-78 years) showed mild improvement in EF after supplementation of vitamin C (SMD: 0.25; 95% CI: 0.01, 0.48). In subgroup analyses, bigger effects were observed in older people > 56 years (Table 3.3). Furthermore, the authors reported significant positive correlation between age and the effect of vitamin C supplementation on EF (Beta: 0.023, 95%

CI: 0.001 to 0.05). However, all the three meta-analyses were associated with high levels of heterogeneity between the included studies [Montero *et al.* (2014): $I^2 = 47\%$; Ashor *et al.* (2014a): $I^2 = 54\%$; (Ashor *et al.*, 2015b): $I^2 = 40.5\%$). Moreover, the study by Ashor *et al.* (2014a) reported evidence for significant publication bias in their meta-analysis.

3.3.3 *Vitamin C and cardio-metabolic biomarkers*

a. Vitamin C and glycaemic control: Analysis of data from five clinical trials (Tabatabaei-Malazy *et al.*, 2014) involving individuals with type 2 diabetes demonstrated significant improvement in fasting blood glucose concentration after vitamin C supplementation (SMD: -20.59; 95% CI: -40.77, -0.40). However, in a recent meta-analysis of 23 trials (998 participants with and without diabetes) (Ashor *et al.*, 2017) showed no significant effects of vitamin C supplementation on glucose concentration (WMD: -0.09 mmol/L; 95% CI: -0.34, 0.18). Nevertheless, subgroup analyses (Ashor *et al.*, 2017) revealed larger effects in diabetics (WMD: -0.44 mmol/L; 95% CI: -0.81, -0.07) and in longer duration studies (> 30 days) (WMD: -0.53 mmol/L; 95CI: -0.97 to -0.10). Moreover, meta-regression analyses showed bigger effects in longer duration studies (β: -0.01, 95% CI: -0.018 to -0.001), higher BMI (β: -0.05, 95% CI: -0.10, -0.01) and in studies with higher baseline plasma concentration of glucose (β: -0.17, 95% CI: -0.29, -0.04).

Both Tabatabaei-Malazy *et al.* (2014) and Ashor *et al.* (2017) showed no significant effects of vitamin C supplementation on insulin and HbA1c concentrations (Table 3.3). However, in a subgroup analysis, (Ashor *et al.*, 2017) revealed significant improvement in fasting insulin concentration following vitamin C supplementation (WMD: -13.63 pmol/L; 95% CI: -22.73 to -4.54). Moreover, in meta-regression analyses, the observed effect size increased with age (β: -0.69, 95% CI: -1.29 to -0.11), in those with higher BMI (β: -3.31, -6.72 to -0.10) and higher baseline plasma glucose concentration (β: -3.65, -7.46 to -0.15). Using data from 3 studies, one meta-analysis (Khodaeian *et al.*, 2015) investigated the effect of vitamin C supplementation on insulin sensitivity (homoeostasis model assessment) index in type 2 diabetes patients and concluded that vitamin C had no effect (SMD: -0.15; 95% CI: -0.49, 0.19).

Table 3.3: Summary of the results of the 10 systematic reviews and meta-analyses of vitamin C and cardiovascular biomarkers included in this umbrella review

Outcome	Study	Number of trials	Effect size	Heterogeneity	Subgroup analysis	Meta-regression analysis	Publication bias
Arterial stiffness	Ashor et al. 2014	10	SMD= -0.16 (-0.42 to 0.11)	I^2 (%)= 29.2	NR	NR	NR
Blood pressure	Juraschеck et al. 2012	29	Systolic BP (WMD): -3.84 (95% CI: -5.29 to -2.38) diastolic BP (WMD): -1.48 (95% CI: -2.86 to -0.10)	Systolic BP: I^2 (%) = 69 diastolic BP: I^2 (%) = 81	Higher effect in < 50 years old: WMD: -5.07 (95% CI: -7.22 to -2.92) higher effect in fewer BP measurements (1-2): WMD: -4.02 (95% CI: -5.57 to -2.47) higher effect in larger sample size studies: WMD: -2.69 (95% CI: -4.49 to -0.88)	NR	No
Endothelial function	Montero et al. 2013	6	SMD= 0.28 (95% CI: -0.19 to 0.74)	I^2 (%)= 47	NR	NR	No
	Ashor et al. 2014	44	SMD= 0.50 (95% CI: 0.34 to 0.66)	I^2 (%)= 54	higher effect in experimentally-induced endothelial dysfunction: SMD: 0.87 (95% CI: 0.62 to 1.13) atherosclerotic: SMD: 0.84 (95% CI: 0.41 to 1.26) diabetics: SMD: 0.52 (95% CI: 0.21 to 0.82)	Significant correlation between vitamin C dose and the magnitude of effect on EF (β: 0.00011, 95% CI: 0.00001 to 0.00021)	Yes
	Ashor et al. 2015	17	SMD= 0.25 (95% CI: 0.01 to 0.48)	I^2 (%)= 40.5	higher effect in older age group > 56 years SMD: 0.58 (95% CI: 0.16 to 0.99)	Significant positive correlation between age and the effect of vitamin C supplementation on EF (β: 0.023, 95% CI: 0.001 to 0.05)	No
Glucose concentration	Tabatabaei-Malazy et al. 2014	5	SMD= -20.59 (95% CI: -40.77 to -0.4)	Cochrane Q test (P = 0.69)	NR	NR	No
	Ashor et al. 2017	23	WMD= -0.09 (mmol/L) (95% CI: -0.34 to 0.18)	I^2 (%)= 55.6	Higher effect in diabetics: WMD= -0.44 (mmol/L)(95% CI: -0.81 to -0.07) longer duration studies (> 30 days): WMD= -0.53 (mmol/L) (95% CI: -0.97 to -0.10)	Higher effect in longer duration studies (β: -0.01; 95% CI: -0.018 to -0.001) higher BMI (β: -0.05; 95% CI: -0.10 to -0.01) and higher baseline plasma concentration of glucose (β: -0.17; 95% CI: -0.29 to -0.04)	No
HbA1c	Tabatabaei-Malazy et al. 2014	5	SMD= -0.46 (95% CI: -1.75 to 0.84)	Cochrane Q test (P < 0.0001)	NR	NR	Yes
	Ashor et al. 2017	10	WMD= -0.02 % (95% CI: -0.19 to 0.15)	I^2 (%)= 0	No effect	No effect	No

Insulin concentration	Ashor et al. 2017	9	WMD= -5.92 (pmol/L) (95% CI: -14.29 to 2.46)	I^2 (%)= 27	Higher effect on fasting insulin: WMD= -13.63 (pmol/L) (95% CI: -22.73 to -4.54)	Higher effect with increased age (β: -0.69; 95% CI: -1.29 to -0.11) higher BMI (β: -3.31; 95% CI: -6.72 to -0.10) and higher baseline plasma concentration of glucose (β: -3.65; 95% CI: -7.46 to -0.15)	No
Insulin resistance	Khodaeian et al. 2015	3	SMD= -0.15 (95% CI: -0.49 to 0.19)	I^2 (%)= 35.4	NR	NR	No
HDL-cholesterol	McRae et al. 2008	12	WMD= 1.1 (mg/dL) (-0.2 to 2.3)	NR	NR	NR	Yes
	Ashor et al. 2016	35	WMD= 0.004 (mmol/L) (95% CI: -0.03 to 0.03)	I^2 (%)= 45	Higher effect in diabetics: WMD: -0.06 (95% CI: 0.02 to 0.11)	Greater effect in those with low baseline plasma concentration of vitamin C (β: -0.002; 95% CI: -0.003 to -0.0001)	No
LDL-cholesterol	McRae et al. 2008	11	WMD= -7.9 (mg/dL) (-12.3 to -3.5)	NR	NR	NR	No
	Ashor et al. 2016	29	WMD= -0.09 (mmol/L) (95% CI: -0.28 to 0.11)	I^2 (%)= 85	Higher effect in healthy participants: WMD: -0.32 (95% CI: -0.57 to -0.07)	A trend towards a larger reduction in LDL-C concentration following vitamin C supplementation in participants with low baseline plasma concentration of vitamin C (β: 0.01; 95% CI: -0.004 to -0.03)	No
Total cholesterol	Ashor et al. 2016	37	WMD= -0.10 (mmol/L) (95% CI: -0.22 to 0.01)	I^2 (%)= 57.2	Higher effect in younger participants (≤52 years) : WMD: -0.26 (95% CI: -0.45 to -0.07)	Greater effect in those with higher baseline plasma cholesterol concentration (β: -0.24; 95% CI: -0.36 to -0.11)	No
Triglycerides	McRae et al. 2008	10	WMD= -20.1 (mg/dL) (95% CI: -33.3 to -6.8)	NR	NR	NR	No
	Ashor et al. 2016	35	WMD= -0.03 (mmol/L) (95% CI: -0.13 to 0.08)	I^2 (%)= 72.4	Higher effect in diabetics: WMD: -0.15 (95% CI: -0.30 to -0.002)	Greater effect in those with higher baseline plasma triglyceride concentration (β: -0.17; 95% CI:-0.30 to -0.05)	No

NR: not reported

b. Vitamin C and lipid profile: Two meta-analyses (McRae, 2008; Ashor *et al.*, 2016c) investigated the effects of vitamin C supplementation on lipid profile (i.e. total-, LDL-, HDL-cholesterol and triglyceride (TG) s concentrations). Results from 13 trials (549 participants) (McRae, 2008) demonstrated a significant reduction in LDL-cholesterol (LDL-C) (WMD: -7.9 mg/dL; 95% CI: -12.3, -3.5) and TG (WMD: -20.1 mg/dL; 95% CI: -33.3, -6.8) concentrations. The authors reported no significant effects on HDL-C (WMD: 1.1 mg/dL; 95% CI: -0.2 to 2.3). In contrast, Ashor *et al.* (2016c) reported no significant effects on total cholesterol (WMD: -0.10 mmol/L; 95%CI: -0.22 to 0.01), LDL-C (WMD: -0.09 mmol/L; 95%CI: -0.28 to 0.11), HDL-C (WMD: 0.004 mmol/L; 95%CI: -0.03 to 0.03) and TG (WMD: -0.03 mmol/L; 95%CI: -0.13 to 0.08) concentrations. However, in subgroup analyses, the authors revealed a significant improvement of lipid profile in diabetics (HDL-C and TG), healthy (LDL-C) and younger (≤ 52 years) participants (total cholesterol). Additionally, meta-regression analyses demonstrated significantly greater effects of vitamin C supplementation on lipid profile in individuals with lower baseline plasma concentration of vitamin C and higher baseline plasma concentrations of cholesterol and TG (Table 3.3).

3.4 Discussion

3.4.1 *Summary of the findings*

Close inspection of the results from the 10 systematic reviews and meta-analyses included in this umbrella review revealed a weak evidence of the overall effects of vitamin C supplementation on biomarkers of CVD risk. Meta-analyses conducted by different groups often reached different conclusions. For example, two meta-analyses reported positive effects on EF (Ashor *et al.*, 2014a; Ashor *et al.*, 2015b) whereas no significant effects were found in another meta-analysis (Montero *et al.*, 2014). Similar heterogeneous results were observed for glycaemic control and lipid profile (Table 3.3). However, subgroup and meta-regression analyses indicated significant benefits in participants with advanced age, higher BMI and with low vitamin C intakes and higher CVD risk (Table 3.3).

3.4.2 *Mechanism of action of vitamin C*

Vitamin C is an electron donor (reducing agent) whose antioxidant function derives from its ability to reduce oxidized species or oxidant radicals (Padayatty and Levine, 2016). However, vitamin C can also generate free radicals (i.e. have a pro-oxidant function) by donating electrons to metals such as copper or iron (Halliwell, 1996).

The plasma vitamin C concentration of healthy individuals can vary over a wide range but is typically 30-60μM (Korantzopoulos and Galaris, 2003). Some pharmacokinetics studies have shown that a dose of 200mg/day is sufficient to raise the plasma level of vitamin C to 100-120 μM (Levine *et al.*, 1996) . However, this relationship between ingested dose of vitamin C and plasma vitamin C concentration is non-linear. Oral doses of vitamin C in excess of 400 mg/day do not result in additional increases in plasma vitamin C concentration (Levine *et al.*, 2001). At plasma vitamin C concentrations of < 70μM, the kidneys reabsorb all the filtered ascorbic acid via SVCT transporters. However, with vitamin C concentrations in excess of 70 μM, ascorbate is excreted in urine in progressively greater amounts (Duconge *et al.*, 2008).

In vitro studies demonstrated that mM concentrations of vitamin C in plasma are required to prevent NO from reacting with $O_2^{\cdot-}$ (Frei, 1999). The rate constant for the reaction of vitamin C with $O_2^{\cdot-}$ is 2×10^5 $M^{-1} \times s^{-1}$, while the rate constant of reaction of $O_2^{\cdot-}$ with NO is 1.7×10^{10} $M^{-1} \times s^{-1}$ (Jackson *et al.*, 1998). These studies question the antioxidant capacity of the relatively small concentrations of vitamin C in plasma under normal physiological conditions (Korantzopoulos and Galaris, 2003). The counter-argument is that the relatively high intracellular concentrations of vitamin C (1-5 mM in leukocytes, 1-4 mM in platelets) may indicate its potential to act as an antioxidant (Michels *et al.*, 2013). Moreover, the pleotropic action of vitamin C cannot be reduced to the mere interaction with $O_2^{\cdot-}$ (Korantzopoulos and Galaris, 2003). Vitamin C, besides being a powerful antioxidant, has a broad spectrum of biological functions (Lykkesfeldt *et al.*, 2014). Vitamin C catalyses the hydroxylation of lysine and proline residues in procollagen chains which is regarded as the building blocks for the mature collagen. Moreover, vitamin C serve as an electron donor for a variety of enzymes involved in carnitine and norepinephrine biosynthesis, tyrosine metabolism and peptide hormone amidation. Moreover, the hydroxylation of hypoxia inducible factor 1α by vitamin C regulates the transcription of several genes involved in homeostasis, angiogenesis and cell proliferation (Lykkesfeldt *et al.*, 2014).

3.4.3 ***Evidence from epidemiological studies***

Epidemiological studies support the beneficial effects of vitamin C on cardiovascular outcomes (Moser and Chun, 2016). In the NHANES I Epidemiological Follow-Up Study cohort (11,348 American adults aged 25-74), vitamin C intake was found to be inversely related with all cardiovascular diseases (males, standardized mortality ratio [SMR]: 0.58, 95% CI: 0.41, 0.78; females, SMR: 0.75, 95% CI: 0.55, 0.99) (Enstrom *et al.*, 1992). Another cohort of 85,118 female nurses followed up for 16 years for the incidence of CHD revealed a significant inverse

relationship between total intake of vitamin C and risk of CHD (relative risk [RR]: 0.73, 95% CI: 0.57, 0.94) (Osganian *et al.*, 2003). However, a subgroup analysis showed that supplementation rather than intake of vitamin C from the diet was strongly associated with the reduction in CHD risk (supplemental vitamin C, RR: 0.72; 95% CI 0.61, 0.86; dietary vitamin C, RR: 0.86; 95% CI 0.59, 1.26) (Osganian *et al.*, 2003). Further confirmation has come from studies measuring plasma ascorbic acid concentration (more accurate estimation of vitamin C intake in comparison with questionnaire-based surveys). In NHANES II, serum ascorbic acid was measured and participants followed up for 14-16 years (Loria *et al.*, 2000). Men in the lowest (< 28.4 μmol/L) compared with the highest (≥ 73.8 μmol/L) serum ascorbate quartile had a 57% higher risk of dying from any cause (RR: 1.57; 95% CI: 1.21, 2.03) and a 62% higher risk of dying from cancer (RR: 1.62; 95% CI: 1.01, 2.59) (Loria *et al.*, 2000). Vitamin C deficiency (plasma ascorbate < 11.4 μmol/l) in a random cohort of 1605 Finnish men aged 42, 48, 54, or 60 was found to be significantly associated with increased risk of acute myocardial infarction (RR: 3.5; 95% CI: 1.8, 6.7) (Nyyssonen *et al.*, 1997).

Four years follow-up of a British cohort of men and women aged 45 to 79 years revealed an inverse association of plasma vitamin C concentration with cardiovascular mortality (Khaw *et al.*, 2001). The investigators claimed that 20 μmol/L increases in plasma ascorbic acid were associated with about 20% reduction in all-cause mortality (Khaw *et al.*, 2001). Further analyses from the above cohort showed a significant inverse correlation of plasma ascorbic acid with systolic, diastolic BP (Boekholdt *et al.*, 2006), glucose (Sargeant *et al.*, 2000) and C - reactive protein concentrations (Boekholdt *et al.*, 2006) and positive correlation with HDL concentration (Boekholdt *et al.*, 2006). Meta-analysis of 15 prospective cohort studies (374,488 participants with a median follow-up of approximately 10 years) showed a significant reduction of CHD risk in the highest tertile for vitamin C intake (RR: 0.84; 95% CI, 0.73–0.95) (Ye and Song, 2008). However, subgroup analyses showed a lack of effect of vitamin C supplementation on CHD risk (Ye and Song, 2008).

3.4.4 ***Evidence from large RCTs***

RCTs of antioxidant vitamins supplementation have reported contradictory results (Cordero *et al.*, 2010). A meta-analysis of 50 RCTs (294,478 participants) showed that supplementation with vitamins and antioxidants was not associated with lower risk for major cardiovascular events (RR: 1.00, 95%CI: 0.98, 1.02) (Myung *et al.*, 2013). Moreover, subgroup analysis including RCTs supplementing vitamin C (7 trials) showed lack of effects on major cardiovascular events (RR: 0.99, 95%CI: 0.94, 1.04) (Myung *et al.*, 2013). However, we cannot

rule out the effects of vitamin C supplementation, due to the fact that all of the seven trials included in that subgroup analysis used a combination of vitamin C and other antioxidants supplementation (Mark *et al.*, 1996; Brown *et al.*, 2001; You *et al.*, 2001; 2002; Waters *et al.*, 2002; Hercberg *et al.*, 2004; Sesso *et al.*, 2008).

In vitro studies have demonstrated that vitamin C can regenerate oxidized vitamin E leading to the restoration of its antioxidant capacity (Traber and Stevens, 2011). This potentially beneficial interaction between the two vitamins provides an underpinning mechanism in support of the design and conduct of RCTs testing the efficacy of combined vitamins C and E supplementation on cardiovascular and metabolic outcomes (Cook *et al.*, 2007; Sesso *et al.*, 2008). However, these studies failed to show beneficial effects of the combined supplements on the investigated outcomes. Recently conducted meta-analysis has showed that, in contrast with single supplementation, the co-administration of vitamin C and E did not improve EF (vitamin C: SMD: 0·25, 95% CI 0·02, 0·49; vitamin E: SMD: 0·48, 95% CI 0·23, 0·72; combined: SMD: 0·12, 95% CI 20·18, 0·42) (Ashor *et al.*, 2015b). Moreover, *post hoc* analysis of the WAVE study showed that combined vitamin C and E supplementation exacerbated significantly cardiovascular outcomes in a subpopulation with the haptoglobin 2-2 genotype.

In transgenic animals with the haptoglobin 2-2 genotype, Asleh and Levy reported that the co-administration of vitamin C mitigated the protective effect of vitamin E on high density lipoprotein (HDL) (Asleh and Levy, 2010). Human mechanistic studies have found that adding vitamin C to vitamin E may not cause any further reduction in oxidative stress biomarkers (Dietrich *et al.*, 2002). Similarly, in vitro experimental studies demonstrated that the synergistic action of vitamin C and E disappeared under anaerobic conditions and may be converted to a pro-oxidant effect (Kadoma *et al.*, 2006a). In these studies, monitoring the rate of alpha-tocopheroxyl radical formation in the presence of co-antioxidants such as ascorbate revealed that the rate of alpha-tocopheroxyl radical formation was enhanced with higher concentrations of vitamin E or under anaerobic conditions (Fujisawa *et al.*, 2006; Kadoma *et al.*, 2006b).

3.4.5 ***Beneficial effects of vitamin C in certain populations***

Subgroup and meta-regression analyses revealed beneficial effects in subpopulations with certain phenotypic characteristics such as younger (Juraschek *et al.*, 2012; Ashor *et al.*, 2016c), older age (Ashor *et al.*, 2015b; Ashor *et al.*, 2017), obese (Ashor *et al.*, 2017), having lower vitamin C concentrations (Ashor *et al.*, 2016c) or having higher risk of CVD (Ashor *et al.*, 2014a; Ashor *et al.*, 2016c; Ashor *et al.*, 2017).

Older people are more likely to have inadequate micronutrient intake and so may receive greater benefit from vitamin C supplementation (Brubacher *et al.*, 2000). Inadequate intake may be exacerbated by a reduced capacity for vitamin C absorption with age (Brubacher *et al.*, 2000; Visioli and Hagen, 2007). Furthermore, older people may require more vitamin C than do younger adults to reduce the greater oxidative stress due to age-related mitochondrial dysfunction (Puca *et al.*, 2013); however, the quantitative needs for vitamin C, and for other micronutrients, in older populations are poorly understood. In the SU.VI.MAX Study, Hercberg *et al.* (2004) found that those with lower baseline concentrations of vitamin C and beta-carotene benefitted more from supplementation with antioxidant vitamins and minerals. Furthermore, supplementation with multivitamins for 6 years in a population with high prevalence of micronutrient deficiency improved cerebrovascular disease mortality significantly (Mark *et al.*, 1996). These studies support the concept that individual vitamin status may determine the magnitude of the effect of antioxidant vitamin supplementation on EF and, therefore, explain some of the inter-individual variation in response to such supplementation.

Some investigators question the validity of the conclusions derived from the big RCTs regarding the lack of any beneficial effects of vitamin C supplementation (Lykkesfeldt and Poulsen, 2010; Michels and Frei, 2013). Participants involved in diet-related RCTs are usually healthy-motivated individuals who eat balanced diets and maintain optimal body weight. Therefore, these individuals have overall a lower disease risk and better nutritional status (Michels and Frei, 2013). Moreover, in diet-related studies of vitamin C, there no true placebo group. The control group (non-supplemented group) continues to obtain vitamin C from their diet over the study period (Michels and Frei, 2013). Additionally, failure to measure vitamin C status at baseline and over the study duration may question the effectiveness of the supplements in these studies (Lykkesfeldt and Poulsen, 2010). In addition, there is wide inter-individual variation in vitamin C levels (e.g. age, smoking status, BMI, socioeconomic status and genetic polymorphisms), which may considerably affect plasma concentrations and, consequently, the physiological response to supplemental vitamin C (Frei *et al.*, 2012; Michels and Frei, 2013; Michels *et al.*, 2013).

3.4.6 ***Limitations***

The main limitations of this umbrella review are related to the characteristics of the trials included in each meta-analysis such as small sample size, intervention of short duration, different recruitment strategies and measurement protocols. This large heterogeneity represented a serious limitation which impeded the conduction of an umbrella meta-analysis.

3.4.7 ***Conclusions***

Vitamin C seems to significantly improve cardiovascular biomarkers in selected populations (advanced age, obesity, vitamin C deficiency or presence of higher CVD risk). However, these results are still preliminary due to important limitations of the currently available evidence from RCTs. Future trials should take into account these limitations to achieve greater efficacy and sustainability of the physiological effects derived from vitamin C supplementation.

Chapter 4. Impact of inorganic NO_3^- on adverse cardiometabolic effects induced by acute hyperglycaemia in younger and older obese participants

4.1 Introduction

Post-prandial hyperglycaemia (PPH) is a major predictor of CV mortality (Mah and Bruno, 2012). In a Japanese cohort followed up for 7 years, the presence of impaired glucose tolerance (IGT) was associated with a higher risk of death from CVDs (Tominaga *et al.*, 1999). Similarly, in a 10-year cohort of 2620 middle-aged participants, participants with IGT had a significantly higher risk for CHD (hazard ratio [HR]: 1.49, 95% CI: 0.95, 2.34), CVD mortality (HR: 2.34; 95%CI: 1.42, 3.85) and all-cause mortality (HR: 1.65; 95%CI: 1.13, 2.40) (Qiao *et al.*, 2003).

Heightened intracellular glucose concentration increases free radical production secondary to uncoupling of mitochondrial oxidative respiration (Siervo *et al.*, 2011a). It has been postulated that the free radicals associated with PPH cause endothelial dysfunction by decreasing NO availability. These reactive oxygen species deplete arginine, oxidise BH4 and enhance ADMA, consequently causing eNOS uncoupling which increases free radicals and reduces NO availability (Mah and Bruno, 2012).

Ageing is associated with key morphological and functional changes including increased risk of central obesity, insulin resistance and endothelial dysfunction (Figure 4.1) (Ahima, 2009). The global prevalence of obesity is rising and the consequential burden on public health may become unsustainable if effective lifestyle and nutritional prevention strategies are not developed and implemented (Nguyen and Lau, 2012; Ng *et al.*, 2014). Obesity is a major determinant of numerous life-threatening co-morbidities, most notably type 2 diabetes, coronary heart disease and cancer (Haslam and James, 2005). In particular, excess adiposity is associated with increased release of inflammatory cytokines which impairs insulin signalling and glucose disposal as well as predisposing to vascular damage as a result of reduced anti-oxidant capacity and NO availability (Martyn *et al.*, 2008; Bakker *et al.*, 2009).

NO is a gaseous signalling molecule that plays an essential role in regulating systemic physiological functions (e.g. vascular tone, muscular performance, immune function, and neurotransmission) and NO deficiency has been associated with multiple pathological processes including atherosclerosis, insulin resistance and mitochondrial dysfunction (Yetik-Anacak and Catravas, 2006). Two major pathways contribute to systemic NO formation (Sindler *et al.*, 2014). The endogenous pathway produces NO from L-arginine in the presence

of oxygen and is catalysed by NO synthase (Sindler *et al.*, 2014). The exogenous NO_3^--NO_2^--NO pathway makes a quantitatively smaller contribution to NO formation (Lidder and Webb, 2013). Ingested NO_3^- is reduced to NO_2^- by the commensal bacteria in the oral cavity (Govoni *et al.*, 2008). Upon swallowing, NO_2^- is further reduced in the gastric acidic environment to nitrous acid, which spontaneously decomposes to NO and other bioactive nitrogen oxides (Lidder and Webb, 2013). Additionally, circulating NO_2^- may be converted to NO via numerous NO_2^- reductases such as deoxyhaemoglobin and xanthine oxidoreductase (Sibmooh *et al.*, 2008).

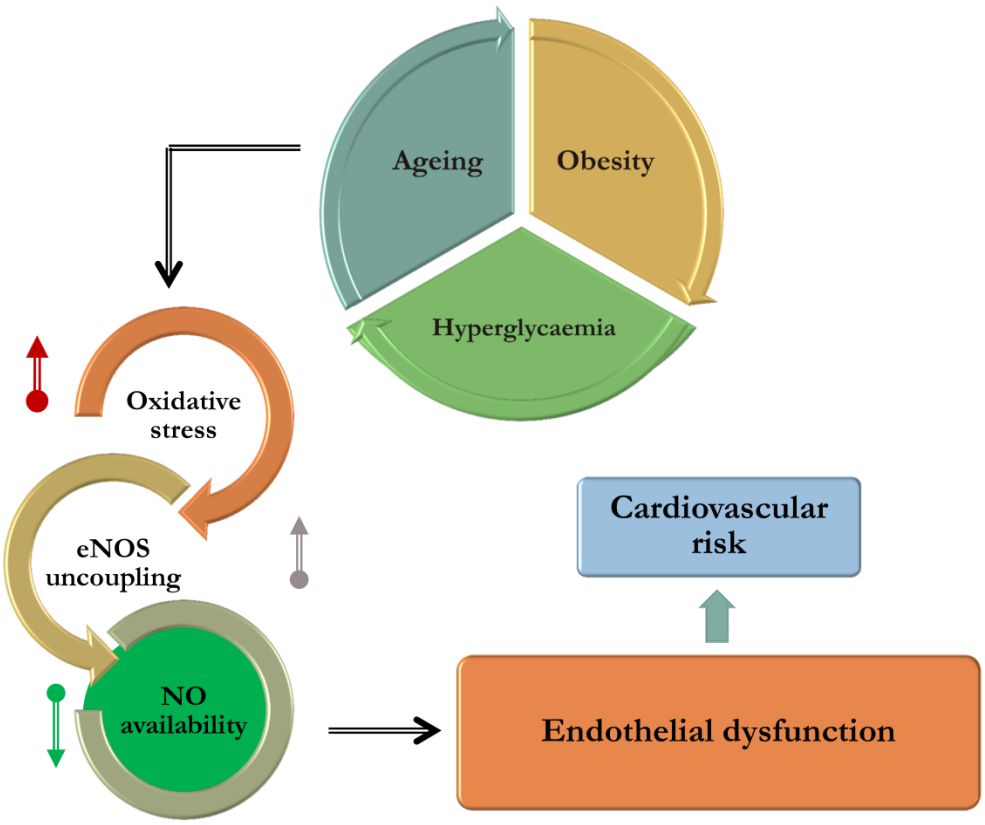


Figure 4.1: The relationship of ageing, obesity and hyperglycaemia with endothelial dysfunction

Recent meta-analyses have showed that supplementation with NO_3^--rich beetroot juice or inorganic NO_3^- improves systolic blood pressure (BP) and endothelial function (Siervo *et al.*, 2013; Lara *et al.*, 2015). Moreover, high consumption of NO_3^--rich green leafy vegetables (e.g. rocket, broccoli, cabbage, lettuce and spinach) reduces the risk of developing type 2 diabetes (Carter *et al.*, 2010). The positive effects of inorganic NO_3^- on metabolic functions have been corroborated in animal models of metabolic syndrome and type 2 diabetes and these benefits were linked to reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity

and 5' adenosine monophosphate-activated protein kinase (AMPK) phosphorylation, improved pancreatic β-cell secretion and glucose transporter 4 (GLUT-4) translocation (Carlstrom *et al.*, 2010; Jiang *et al.*, 2014; Khalifi *et al.*, 2015; Peleli *et al.*, 2015).

The current study aimed to test the hypothesis that the administration of inorganic NO_3^- could ameliorate the acute adverse effects of postprandial hyperglycaemia on vascular function in obese participants. We also tested the hypothesis that the response to the NO_3^- administration is age-dependent with potentially greater benefits in older aged than in younger participants.

4.2 Methods

4.2.1 *Participants*

The study group comprised 20 volunteers (13 males, 7 females) with an age range of 18-70 years and a BMI range of 30-40 kg/m^2 who were recruited in the Clinical Ageing Research Unit (CARU), Newcastle University from September 2013 until November 2014. The study was approved by the regional ethics committee of Yorkshire & the Humber - Humber Bridge (REC reference: 13/YH/0253, Appendix A) and was registered with Current Controlled Trials (ISRCTN42776917). All participants were fully informed of the nature and purpose of the study and provided written consent to participate.

4.2.2 *Exclusion criteria*

Participants were excluded if they had medical conditions (i.e. diabetes, cancer or systemic inflammatory disorders) or were taking medications (i.e. organic nitrates, corticosteroids, diuretics, hormonal therapies and weight loss medications) that could interfere with the nutritional intervention and study outcomes. In addition, individuals were excluded if they were smokers, vegetarian, reported alcohol intake greater than 21 U/week for men and 14 U/week for women, or their body weight had changed more than 3 kg within the last month.

4.2.3 *Study design and randomisation*

This was a randomised, cross-over, double-blind, placebo controlled clinical trial. The randomisation was conducted by an independent investigator outside the research team. The preparation and labelling of the two solutions (inorganic NO_3^- and placebo) were performed by the Pharmacy Department of the Newcastle upon Tyne Hospitals (Royal Victoria Infirmary). A member of the CARU Research Facility outside the research team was the guarantor of the

safety and accessibility of the randomisation codes. Inorganic NO_3^- and placebo solutions had the same organoleptic characteristics and had identical volume, colour and presentation.

4.2.4 ***Study protocol***

The study was divided into three phases: screening, intervention and wash-out periods. An initial telephone screening interview was conducted to evaluate eligibility for the study. Eligible participants were invited for their first visit at the research facility early in the morning (~8.30 am) in fasting condition (~12 h after last eating). Participants were asked to follow a 24-h run in period to standardise NO_3^- intake and to refrain from strenuous exercise, limit alcohol and caffeine consumption and not use mouthwash prior to each testing visit. Body weight, height and waist circumference were measured according to standardised protocols. Body fat was assessed by bioelectrical impedance analysis (Tanita BC420 MA, Tanita Corporation, Tokyo, Japan). A cannula was then fitted in an ante-cubital vein while participants were supine.

Baseline measurements were performed. Immediately following collection of the baseline blood sample, participants were given an oral dose of glucose (75g) and they were randomised to receive a weight-adjusted dose of either inorganic NO_3^- or placebo in a cross-over order. Participants were then asked to rest for the next three hours and BP was measured and blood samples were collected at 15, 30, 60, 90, 120, 180 minutes post-intervention. Microvascular function was measured at baseline and 3-hours post-intervention. A wash-out period of seven days was allowed before the second intervention. During the wash-out period, participants were asked to resume their habitual lifestyle and diet (including caffeine and alcohol consumption, if relevant). After the 7-day wash-out period, participants returned for the second visit having been asked to follow a similar 24-h run in period prior to the start of the second assessment visit. Measurements were repeated in the same order and participants received the cross-over intervention. Each participant was asked to complete a food frequency questionnaire [FFQ] to assess food intake over the last month. Physical activity and NO_3^- intake (Appendix B) over the week prior to each visit were documented through additional questionnaires. A summary of the study protocol is provided in Figure 4.2.

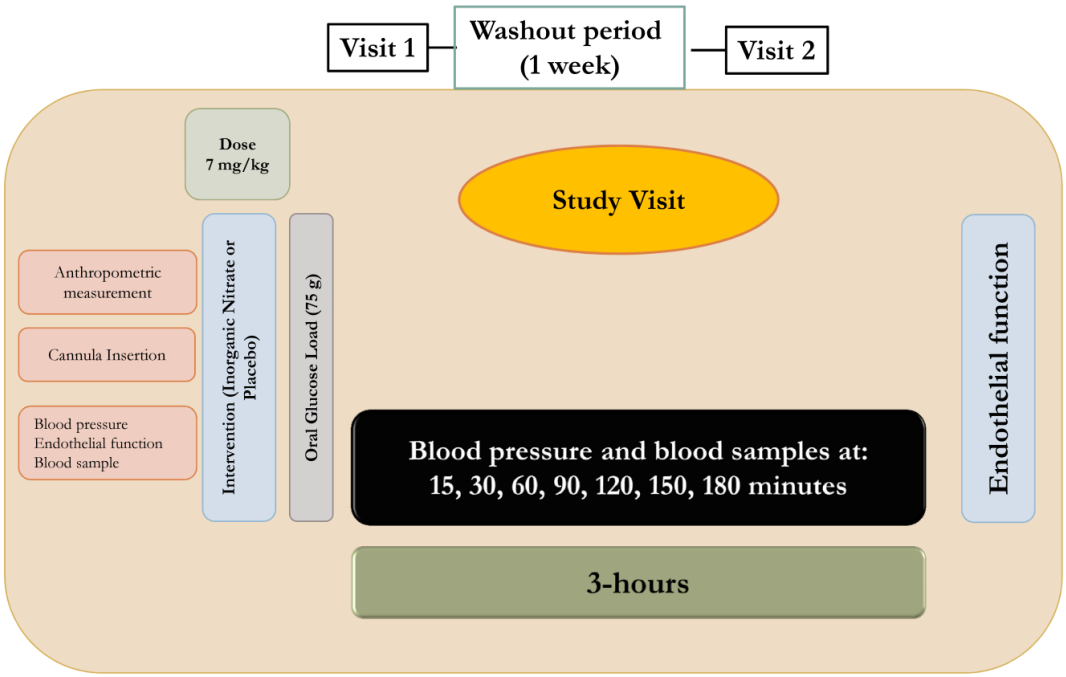


Figure 4.2: Study protocol conducted at each study visit

4.2.5 *Nutritional supplements*

Participants were randomised to receive either 1) NO_3^- supplementation (oral dose of 7.0 mg [0.1 mmol/kg] of potassium NO_3^- per kilogram of body weight in sterile water) or 2) placebo (7.0 mg of potassium chloride per kilogram of body weight in sterile water). Participants first drank the glucose solution (75g anhydrous glucose dissolved in 250 ml of NO_3^--free water) which was followed immediately by the oral administration of a solution of either inorganic NO_3^- or placebo. The NO_3^- dose corresponded to the amount normally found in 150–250 g of a NO_3^--rich vegetable such as spinach, lettuce or beetroot (Larsen *et al.*, 2007). In our trial we used potassium rather than sodium in the placebo and the intervention groups to avoid the cardiovascular adverse effects associated with increased sodium intake (Geleijnse *et al.*, 2003).

4.2.6 *Skin microvascular blood flow*

Laser Doppler Iontophoresis (LDI, Moor Instruments, Axminster, UK) is a non-invasive technique used for the transdermal delivery of charged substances. We used 1% acetylcholine (Ach) to quantify changes in endothelial-dependent microvascular blood flow by measuring non-invasively the increase in erythrocyte flux after Ach application. Forearm skin erythrocyte flux was measured for 5 minutes prior to iontophoresis (baseline), for 5 minutes during Ach delivery by iontophoresis (stimulation) and for 5 minutes after the Ach delivery (recovery). All assessments were performed with the participant supine in a temperature-controlled room (22-

24 °C) following an acclimatisation period of 10 minutes. LDI measurements were stored directly onto a PC and later analysed using MoorVMS V3.1 software. Quantification of blood flow was expressed in perfusion units (PU) in relation to an internal standard calibration of the device. The perfusion index was calculated as the ratio of the area under the curve (AUC) for the stimulation period relative to the AUC of the baseline period. Figure 4.3 below showed an example of blood flow recording from study participant.

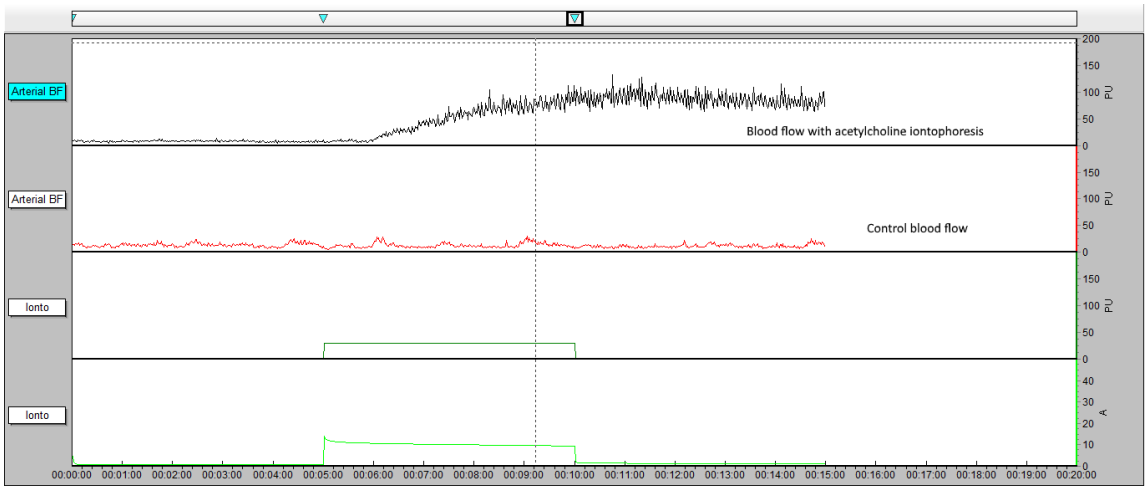


Figure 4.3: Laser Doppler Iontophoresis (LDI) recording from a participant during one of the study visits.

4.2.7 *Blood sample processing and biochemical assays*

Blood samples were stored on ice and processed within 30 minutes of collection. Samples were spun at 4000 x g for 10 minutes at 4°C and serum and plasma aliquots were stored immediately at -80°C until further analyses. These samples were used to measure glucose and insulin concentrations, NO_3^- plus NO_2^- concentrations (NOx) and interleukin 6 (IL-6). Plasma glucose was measured (in the Royal Victoria Infirmary) by standard automated enzymatic methods using an Olympus AU 640 analyser (Olympus, Watford, UK) and fasting insulin (in the Royal Victoria Infirmary) by immunoassay (ELISA; Dako UK Ltd, Ely, UK). NOx were analysed in the School of Agriculture, Food & Rural Development at Newcastle University using gas chromatography mass spectrometry (GC-MS) (Qadir *et al.*, 2013). IL-6 was measured in University of Cambridge Laboratories using high-sensitivity, quantitative ELISA kits (R&D systems, Minneapolis, MN, USA).

The following biomarkers were measured in our laboratory in the Human Nutrition Research Centre, Newcastle University: cGMP, 3 nitrotyrosine (3-NT) and human vascular injury multiplex (P- selectin, E- selectin, ICAM-3 and thrombomodulin).

4.2.8 *cGMP*

cGMP is formed by the action of the enzyme guanylate cyclase on guanosine triphosphate (GTP) (Murad, 1986). It acts as a second messenger for many hormones and molecules in the blood such as insulin, oxytocin, acetylcholine, serotonin and NO (Murad, 1986). I used cGMP Complete ELISA kit (ENZO Life Sciences Inc) for analysing cGMP in plasma samples. This is a competitive immunoassay for the quantitative determination of cGMP. The standards and samples were added to wells coated with goat anti-rabbit (GxR) IgG antibodies. Then we added a blue solution of cGMP conjugated to alkaline phosphatase. This is followed by adding a yellow solution of rabbit polyclonal antibodies to cGMP. During the incubation period, the antibodies will competitively bind to cGMP in the samples or conjugates. When the plate washed, only the bound cGMP remained in the wells. In the next step, we added p-nitrophenyl phosphate (pNpp) substrate solution. This substrate generates a yellow colour when catalysed by the alkaline phosphatase on the cGMP conjugate. The last step, we added the stop solution and read absorbance at 405nm. The amount of signal is indirectly proportional to concentration of cGMP in the sample. Detailed steps followed in conducting the assay are summarised in (Appendix C).

4.2.9 *3 nitrotyrosine*

3 nitrotyrosine considered as an index of oxidative stress (Ischiropoulos, 2009). It is formed by the interaction of reactive nitrogen species (peroxynitrite anion, nitrogen dioxide) with the tyrosine residues of proteins (Radi, 2004). This reaction is considered as a post-translational modification of these proteins with consequent changes in its function (Ischiropoulos, 2009). Moreover, 3 nitrotyrosine concentrations strongly correlated with CVD (Shishehbor *et al.*, 2003). Significantly higher levels of nitrotyrosine were found among patients with CAD (Shishehbor *et al.*, 2003). I used ELISA kits (ab113848, Abcam, Cambridge, USA) to analyse 3 nitrotyrosine in serum samples. This ELISA kits utilizes nitrotyrosine-coated plates and horseradish peroxidase (HRP)-conjugated antibodies to detect 3 nitrotyrosine in the samples. The detailed steps that followed in conducting the assay are summarised in (Appendix C).

4.2.10 *Human vascular injury multiplex (P- selectin, E- selectin, intercellular adhesion molecule 3 [ICAM 3] and thrombomodulin)*

P-, E- selectins, ICAM-3 and thrombomodulin are adhesion molecules secreted mainly from the endothelium during inflammation and vascular damage (Blankenberg *et al.*, 2003). P-, E-selectins and ICAM-3 facilitate the rolling and adhesion of leukocytes to endothelial cells (Dong *et al.*, 1998; Blankenberg *et al.*, 2003). Thrombomodulin secreted from damaged endothelium and it facilitated thrombolysis in the vessel wall (Takano *et al.*, 1990). All these molecules were found to be increased in patients with CVDs (Lind, 2003). The human vascular injury I kit (Meso Scale Discovery [MSD], Gaithersburg, MD, USA) biomarker assay provide a rapid and convenient method for measuring the levels of these biomarkers within a single, small volume sample. These assays are sandwich immunoassays. The plates are pre-coated with capture antibodies on independent and well-defined spots. Multiplex kits are provided on 4-spot plate. Detailed steps followed in conducting the assay are summarised in (Appendix C).

4.2.11 *Statistical analysis*

All statistical analyses were completed using SPSS for Windows (version 22.0; SPSS inc, Chicago, IL, USA). Summary data are presented as means ± SEM. Changes were analysed using paired Student's t tests or repeated-measure analysis of variance (ANOVA) with time and intervention as within-subject factors and age as between-subject factor. Models were checked for sphericity assumptions and multivariate models were applied if these assumptions were violated. If the model was significant, post hoc tests with Bonferroni correction to control for multiple comparisons were conducted. P values < 0.05 were considered to indicate statistical significance.

4.3 Results

4.3.1 *Recruitment and baseline characteristics of the participants*

A total of 20 participants were randomised to the interventions, ten younger (31.4±1.8 years) and ten older (64±1.3 years) participants (Figure 4.4). Laser Doppler Iontophoresis data were complete for all twenty volunteers but biochemical analyses data were obtained from only nineteen participants as we were unable to collect blood samples from one participant in the older group. The interventions were well-tolerated and no adverse effects were reported. Baseline characteristics of the participants (Table 4.1) shows that the younger and older participants were well-matched for anthropometric variables, dietary energy intake and physical activity but systolic BP was significantly higher in older participants ($P = 0.01$).

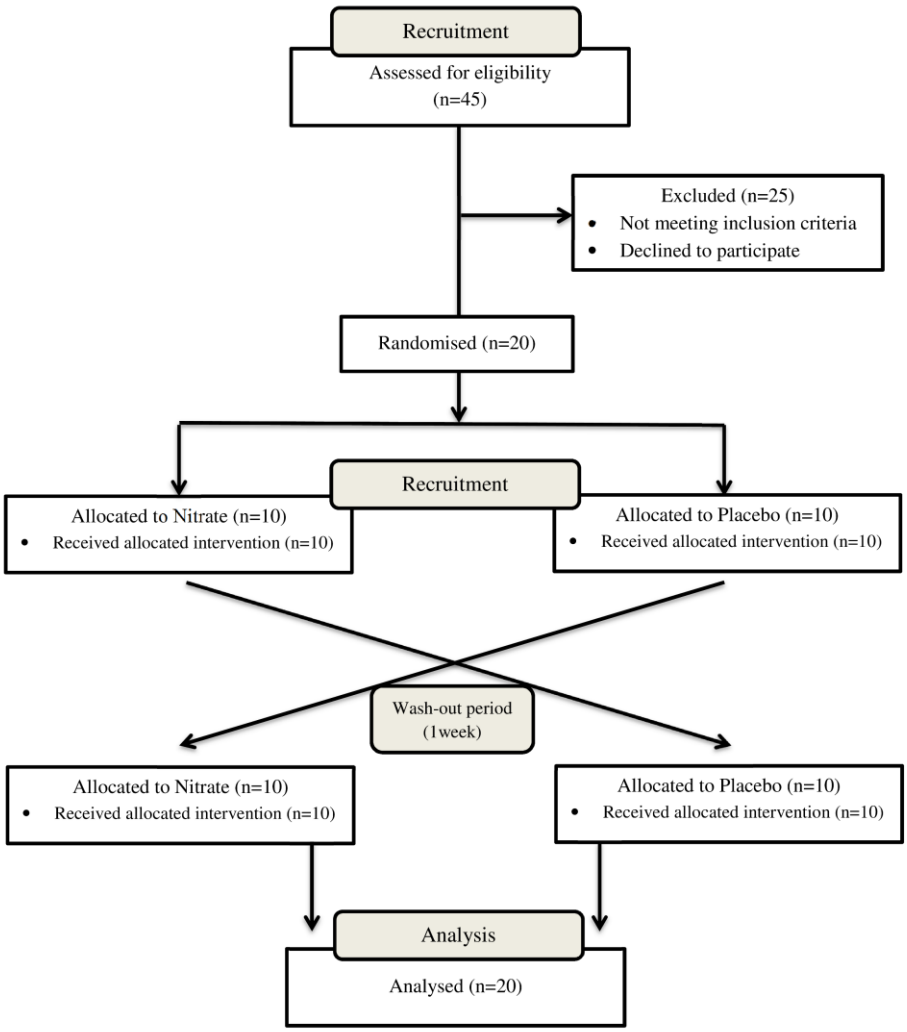


Figure 4.4: Flowchart of recruitment phases

Table 4.1: Baseline characteristics of younger and older participants

	All	Younger	Older	*P* value
Number	20	10	10	
Gender, male/female	13/7	8/2	5/5	
Age, years	47.8±3.9	31.4±1.8	64±1.3	<0.01
Height, cm	169.8±2.1	168.5±2.1	171±3.7	0.56
Body weight, kg	98.7±3.3	95.9±3.9	101.5±5.4	0.41
Body mass index, kg/m^2	34.2±0.8	33.7±0.9	34.7±1.4	0.58
Waist circumference cm	109.9±2.5	106.5±3.8	113.2±3	0.18
Fat mass, kg	36.5±2.1	33±3	40.4±2.5	0.07
Fat mass, %	37.3±1.8	34.2±2.3	40.7±2.5	0.07
Total body water, L	45.3±1.9	46.2±2.2	44.2±3.6	0.63
Heart rate, bpm	60.7±2.6	62.6±2.2	58.8±4.8	0.47
Systolic BP[1], mmHg	125.5±4.1	114.5±2.8	136.4±6.1	0.01
Diastolic BP, mmHg	71.9±2.1	68.8±3.3	75.1±2.4	0.13
Energy intake, kJ/day	11247±1303	11106±2313	11375±1473	0.92
Physical activity, METs/wk[2]	2442±486	2257±440	2627±897	0.72

Data shown as mean±SEM. [1]BP= blood pressure. [2]MET= Metabolic Equivalent of Task

4.3.2 NO_3^-/NO_2^- *(NOx) and cGMP concentrations*

Baseline plasma concentrations of NOx were significantly higher in the younger (246±24 μmol/l) compared with older (168±26 μmol/l) participants (P = 0.04). Plasma NOx did not change between baseline and 3h post-intervention when the placebo was administered (P = 0.91) (Figure 4.5). Following the ingestion of inorganic NO_3^-, plasma NOx reached peak concentration around 2 h post-intervention (young: 798±32 μmol/l; old: 430±48 μmol/l) and there was a significant difference between age groups for plasma NOx concentrations with higher concentrations (~42%, P = 0.01) observed in the younger group. Pairwise comparison demonstrated that, at all times post-inorganic NO_3^- administration, NOx concentration was significantly higher in younger than in older participants. Inorganic NO_3^- did not modify the concentration of cGMP (Figure 4.6) but it was significantly higher in younger compared with older participants (P < 0.01).

4.3.3 *Glucose and insulin concentrations*

At baseline, plasma glucose (Figure 4.7) and insulin (Figure 4.8) concentrations did not differ between placebo and inorganic NO_3^- treatments (P = 0.36). Peak plasma concentrations of

glucose and insulin were reached 60 minutes post-administration of the glucose load ($P < 0.001$).

4.3.4 *Skin microvascular function (Laser Doppler iontophoresis)*

Inorganic NO_3^- supplementation did not affect cutaneous microvascular blood flow in younger or older participants (Table 4.7).

4.3.5 *Biomarkers of endothelial function*

Inorganic NO_3^- supplementation reduced E- and P-selectin concentrations ($P = 0.04$) with greater decline being observed for P-selectin in the younger group ($P = 0.04$) (Figure 4.9, Figure 4.10). Inorganic NO_3^- did not modify the concentrations of thrombomodulin, ICAM-3 (Figure 4.11, Figure 4.12) but thrombomodulin was consistently higher in younger and ICAM-3 was significantly higher in older participants ($P < 0.01$).

4.3.6 *Biomarkers of inflammation and oxidative stress*

Acute induction of hyperglycaemia tended to increase IL-6 concentrations in older compared with the younger participants and this increase tended to be attenuated by inorganic NO_3^- ($P = 0.06$; Figure 4.13). Hyperglycaemia influenced 3-NT concentrations only in older participants whereas concentrations were unaffected in younger participants ($P = 0.08$). In older participants inorganic NO_3^- significantly reduced 3-NT in comparison with placebo ($P = 0.04$) and a significant difference was reached at 180 minutes ($P = 0.01$) (Figure 4.14).

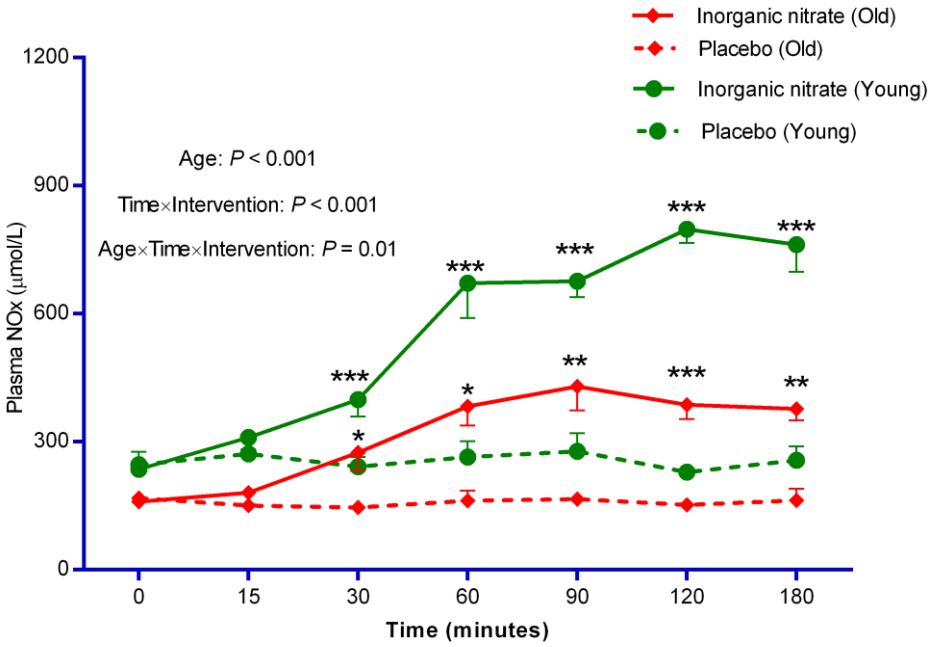


Figure 4.5: Plasma NOx in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, *n*= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA. *Different from placebo intervention within age group. *: *P* < 0.05; **: *P* < 0.01; *: *P* < 0.001.**

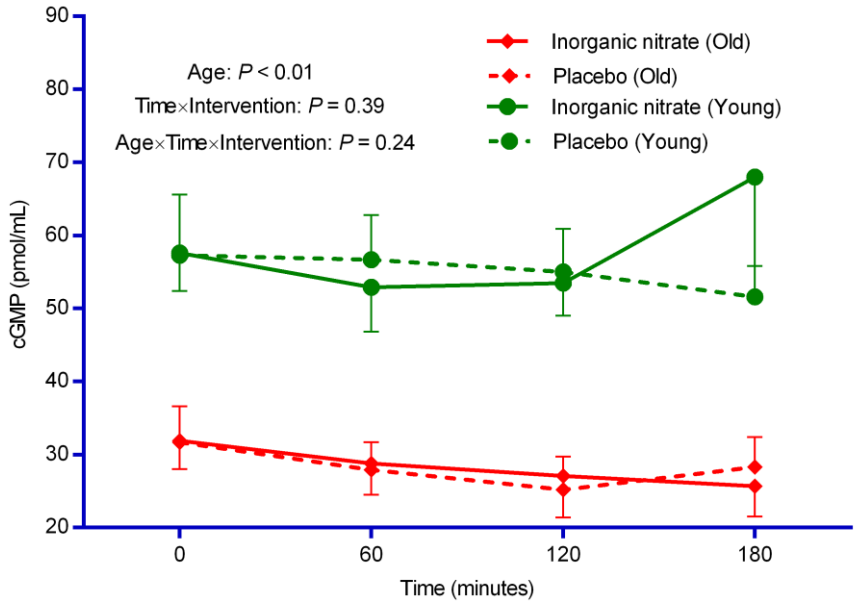


Figure 4.6: Plasma cGMP in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, *n*= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA.

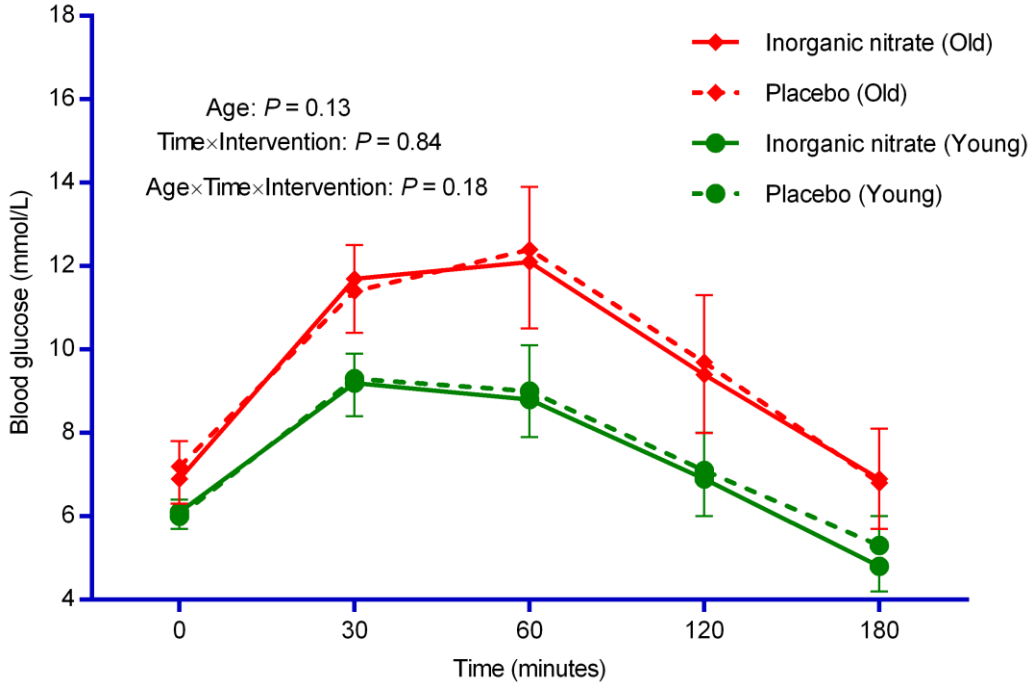


Figure 4.7: Plasma glucose concentrations after a 75 g oral glucose challenge in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, *n*= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA.

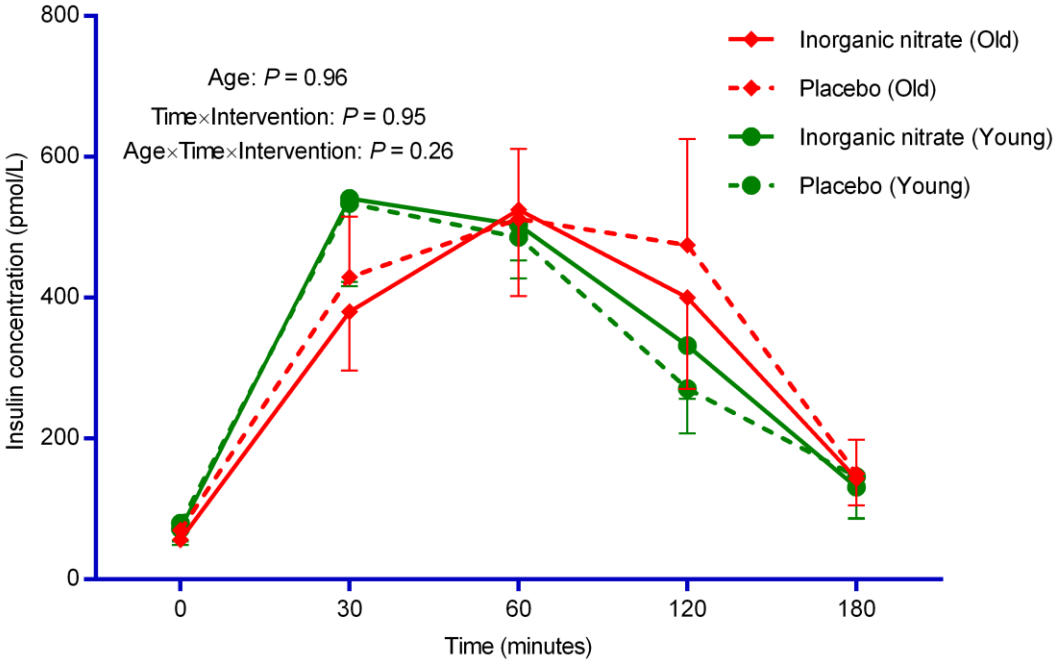


Figure 4.8: Plasma insulin concentrations after a 75 g oral glucose challenge in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, *n*= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA.

Table 4.2: The effects of inorganic NO_3^- supplementation on skin microvascular blood flow in younger and older participants

	All (n=20)				Older (n=10)				Younger (n=10)				
	Dietary NO_3^-		Placebo		Dietary NO_3^-		Placebo		Dietary NO_3^-		Placebo		
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	*P* value
Skin microvascular blood flow													
Baseline flow	9.4±0.9	11.1±1.3	9.8±0.8	12.7±1.6	9.8±1.5	12.1±2.4	10.8±1.2	10.1±1.9	9.1±0.8	10.1±0.9	8.7±0.9	15.2±2.4	0.47
Peak iontophoresis flow	71.4±6.7	56.2±5.3	61.1±7.6	73.8±18.9	82.8±8.8	61.6±9.1	65.9±8.9	49.6±7.9	59.9±9	50.8±5.4	56.2±12.5	98±36.4	0.16
Post-iontophoresis flow	98.9±9.6	86.6±8.6	85.6±11	94.6±21	114±12	104±14	97.1±14	73.7±12	84.3±14	69.4±7.9	74.1±16	116±41	0.12
Perfusion index	8.6±0.9	6±0.7	6.2±0.7	6±1	9.9±1.4	6.5±1.1	5.9±0.6	5.4±0.8	7.3±1.2	5.5±0.8	6.4±1.2	6.7±2	0.90

Values represent mean±SEM, *P* values represents 3-factor repeated-measures (age x time x treatment) ANOVA

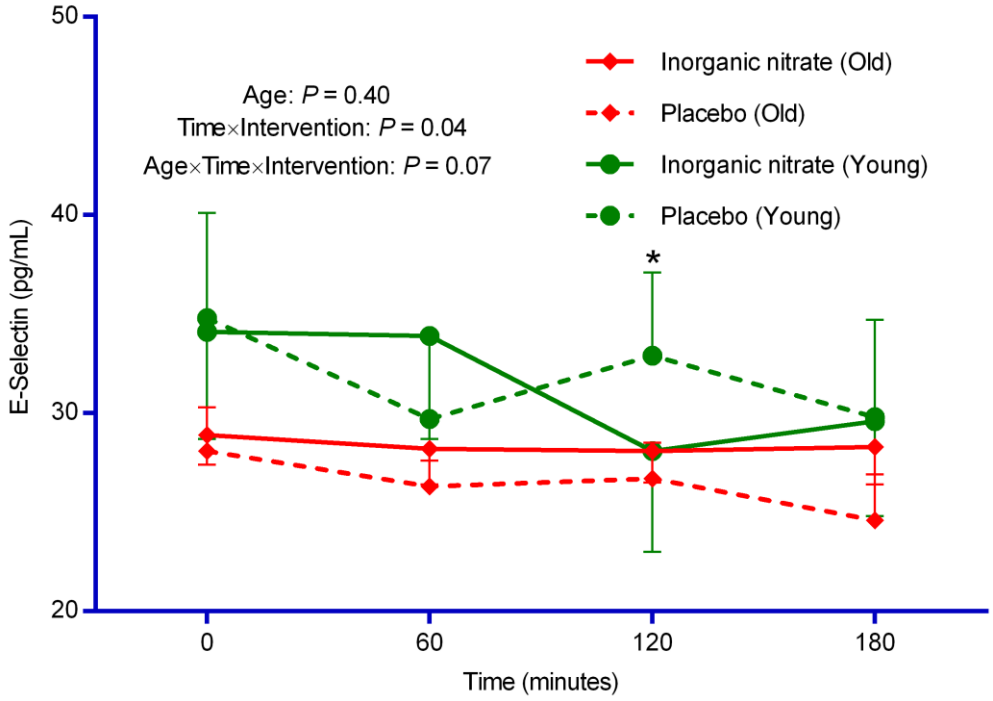


Figure 4.9: Plasma E-selectin concentration after a 75 g oral glucose challenge in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, *n*= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA. Asterisks (*) indicate significant difference from placebo intervention within age group. *: *P* < 0.05

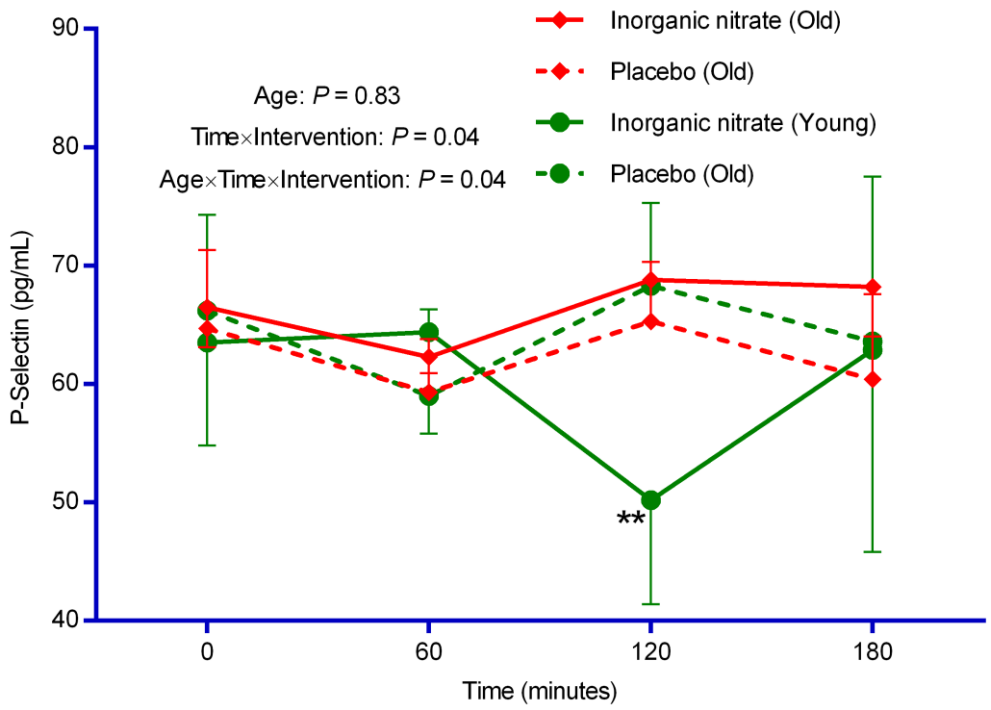


Figure 4.10: Plasma P-selectin concentration after a 75 g oral glucose challenge in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, *n*= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA. Asterisks (*) indicate significant difference from placebo intervention within age group. **: *P* < 0.01.

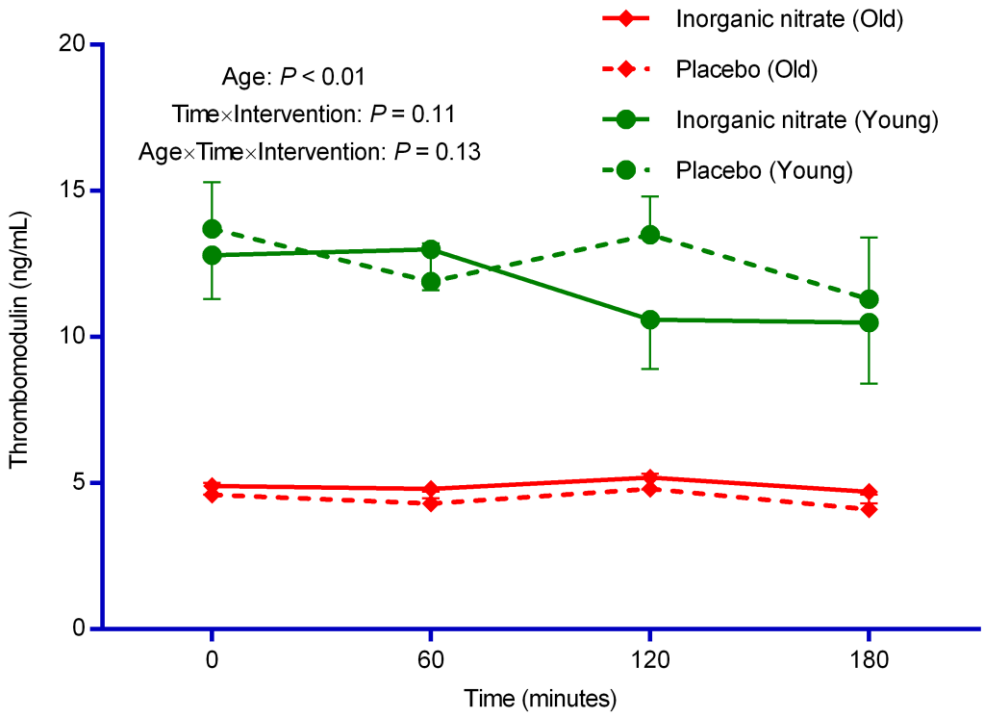


Figure 4.11: Plasma thrombomodulin concentration after a 75 g oral glucose challenge in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, n= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA.

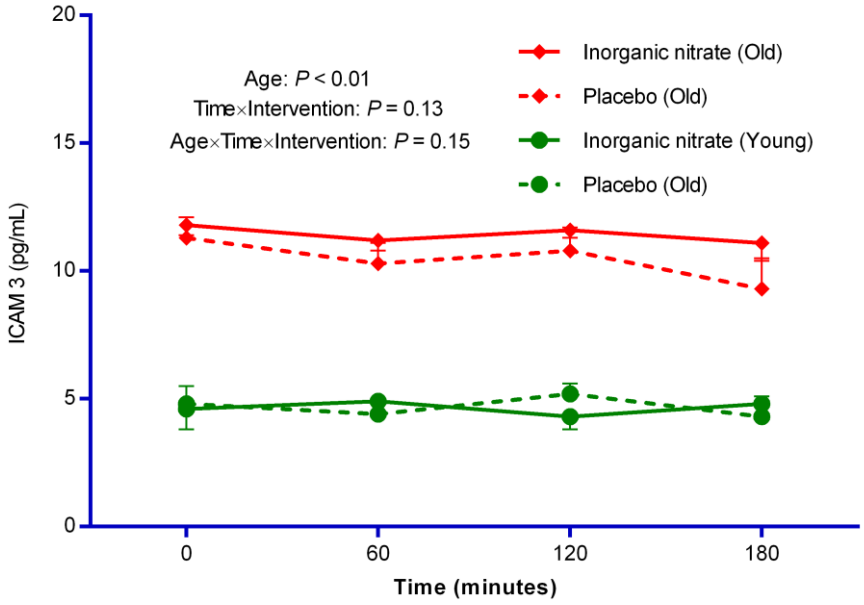


Figure 4.12: Plasma intercellular adhesion molecule-3 (ICAM-3) concentration after a 75 g oral glucose challenge in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, n= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA.

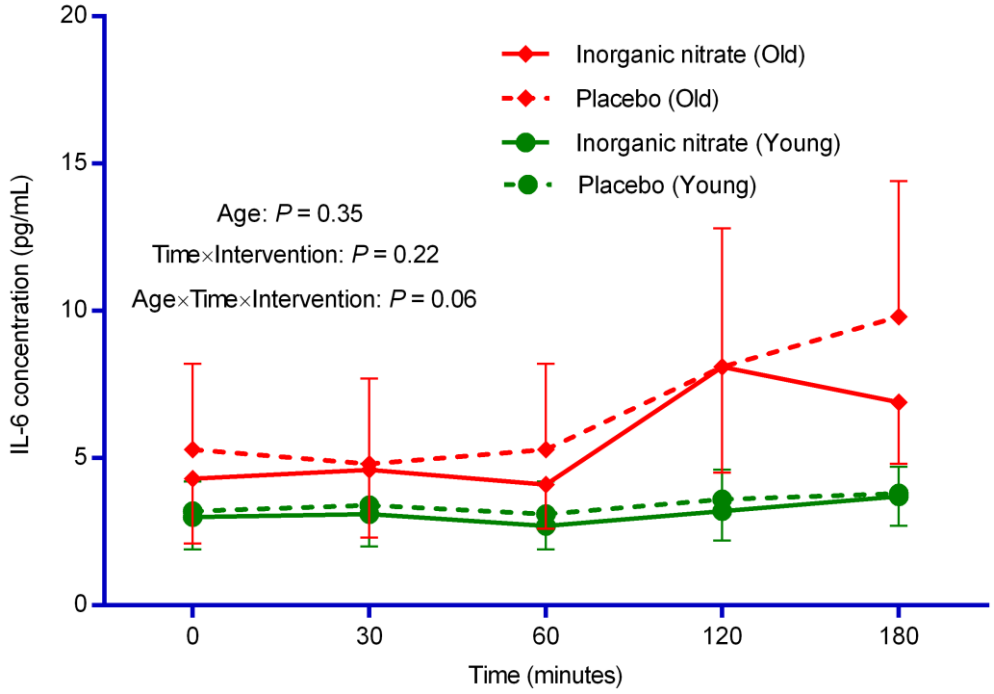


Figure 4.13: Plasma interleukin 6 (IL-6) concentration after a 75 g oral glucose challenge in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, *n*= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA.

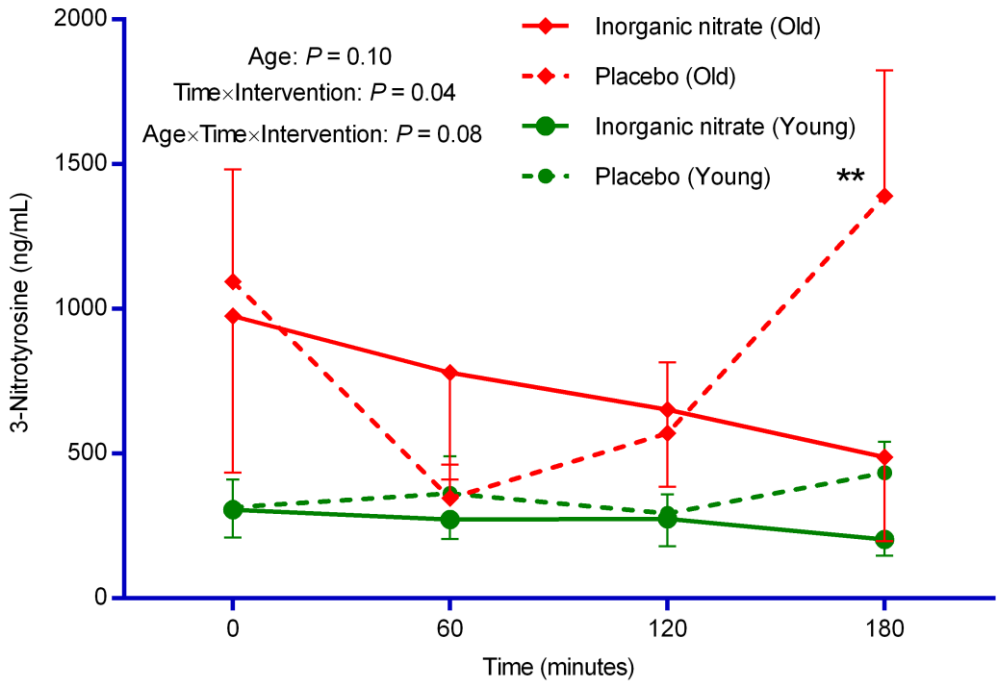


Figure 4.14: Plasma 3-nitrotyrosine concentration after a 75 g oral glucose challenge in younger and older participants given a placebo or a single dose of inorganic NO_3^- (7 mg/kg weight). Values are means ± SEMs, *n*= 9 (old) or 10 (young). Data were analysed with a 3-factor repeated-measures (age x time x treatment) ANOVA. Asterisks (*) indicate significant difference from placebo intervention within age group. **: *P* < 0.01.

4.4 Discussion

4.4.1 *Summary of the findings*

This study tested the hypothesis that inorganic NO_3^- would attenuate the vascular stress associated with acute hyperglycaemia in younger and older obese adults. Additionally, we hypothesized that the response to the NO_3^- administration is potentially of greater benefits in older aged than in younger participants. Inorganic NO_3^- supplementation significantly improved ED and oxidative stress associated with hyperglycaemia. Plasma NOx, cGMP, ICAM-3 and thrombomodulin concentrations were significantly different between age groups. Supplementation with inorganic NO_3^- produced greater improvements in biomarkers of inflammation (IL-6) and oxidative stress (3-NT) in older compared with young participants. Conversely, greater improvement in biomarkers of endothelial function (P- and E-selectin) was observed in younger rather than older participants. However, these changes were not mirrored by improvements in cutaneous microvascular blood flow in either age group.

4.4.2 *Impact of age and obesity on NO metabolites*

Ageing is associated with multiple structural and functional changes of organs and tissues (Mangoni and Jackson, 2004). In the current study, the lower concentration of NOx in older individuals may be related to the lower gastric acidity, reduced intestinal motility and changes in oral and intestinal microflora associated with the ageing process (Schultz *et al.*, 1985; Brownie, 2006; Claesson *et al.*, 2012; Miller *et al.*, 2012). However, in a recently conducted study there were no significant differences in the concentrations of NO_2^- between younger and older non-obese healthy after consumption of 500 ml of beetroot juice which provided 9.4 mmol of NO_3^- (Hughes *et al.*, 2016). Moreover, in our study (Chapter five) we also did not observe a significant difference of NO_3^-/NO_2^- between younger and older non-obese individuals. Therefore, we may speculate that obesity and hyperglycaemia may have significant impact on the concentrations of NO metabolites. However, a recent study reported non-significant correlation between NO_3^-/NO_2^- concentrations and BMI in men (NO_3^-: r = -0.13; P = 0.59 and NO_2^-: r = -0.30; P = 0.20) and women (NO_3^-: r = -0.211; P = 0.37 and NO_2^- : r = 0.34; P = 0.14) (Baiao Ddos *et al.*, 2016). Nevertheless, the authors of that study did not report the BMI range of the included participants. Therefore, it might be underpowered to detect a statistically significant difference between obese and non-obese individuals.

4.4.3 *Hyperglycaemia and EF*

Previous studies showed that PPH significantly deteriorates EF (Sena *et al.*, 2013). For example, Mah *et al.* (2011) observed 40% reduction in FMD in healthy young adults subjected to acute hyperglycaemia. Oxidative stress secondary to mitochondrial dysfunction is the major culprit of hyperglycaemia-induced endothelial dysfunction (Bakker *et al.*, 2009). Hyperglycaemia may exacerbate superoxide radical production in the mitochondria to levels that exceed the endogenous antioxidant capacity (Mah and Bruno, 2012). In addition, oxidative stress deteriorates EF by enhancing eNOS uncoupling through several mechanisms such as oxidation of BH4, arginase-mediated reduction in arginine bioavailability and increased concentration of ADMA (Bakker *et al.*, 2009). eNOS uncoupling may further increase the generation of free radicals and contribute to the damaging effects of a hyperglycaemic state (Carlstrom *et al.*, 2010; Omar *et al.*, 2015).

In addition to oxidative stress, hyperglycaemia is strongly associated with increased inflammation in the vascular wall (Mah and Bruno, 2012). Exposure of human umbilical vein endothelial cell (HUVEC) to 30mM glucose significantly enhanced the expression of ICAM, VCAM, E-selectin, and IL-6 (Piconi *et al.*, 2004). The mechanism of hyperglycaemia-induced inflammation might be related to an increase in the expression of NF-κB in hyperglycaemic conditions (Ho *et al.*, 2006). Furthermore, in human setting, oral glucose challenge of healthy and patients with diabetes revealed significant rise in inflammatory markers such as CRP, E-selectin, ICAM and VCAM (Festa *et al.*, 2002; Ceriello *et al.*, 2004; Konukoglu *et al.*, 2008).

4.4.4 *Anti-inflammatory and antioxidant effects of inorganic NO_3^-*

I hypothesised that supplementation with inorganic NO_3^- would ameliorate some of the adverse effects of acute hyperglycaemia through its anti-inflammatory and antioxidant effects. These may involve scavenging of superoxide, enhanced mitochondrial efficiency or reduced generation of reactive oxygen species (ROS) by xanthine oxidase, NADPH oxidase or cytochrome P450 systems (Carlstrom *et al.*, 2011; Larsen *et al.*, 2011; Montenegro *et al.*, 2011; Peleli *et al.*, 2015; Yang *et al.*, 2015). Treatment of Sprague-Dawley hypertensive rats for eight to 11 weeks with NO_3^- can significantly reduce biomarkers of oxidative stress such as malondialdehyde, F_2-isoprostanes and 8-hydroxy-2-deoxyguanosine (Carlstrom *et al.*, 2011). In addition, NO_3^- administration significantly reduced superoxide production and improved metabolic profile in adenosineA_{2B} receptor knockout mice (Peleli *et al.*, 2015). Similarly supplementation of NO_2^- to mouse model of oxidative stress (superoxide dismutase-1

knockouts) can significantly reduce free radical production by inhibiting NADPH oxidase (Gao *et al.*, 2015).

We observed that acute hyperglycaemia increased inflammatory (IL-6) and oxidative stress (3-NT) biomarkers and that the co-administration of inorganic NO_3^- blunted the rise in these biomarkers in older participants. Two studies have tested the link between inorganic NO_3^- supplementation and oxidative stress in healthy participants (Larsen *et al.*, 2014; Velmurugan *et al.*, 2016). Both studies found a non-significant effect of inorganic NO_3^- on oxidized low density lipoproteins (Velmurugan *et al.*, 2016), total oxidative capacity (Larsen *et al.*, 2014) and plasma isoprostanes (Larsen *et al.*, 2014). Velmurugan *et al.* (2016) also observed a non-significant effect of beetroot juice supplementation on biomarkers of inflammation (i.e. C reactive protein and chemokine (CXC motif) ligand 1 [CXCL1]). Several reasons could explain the differences across studies including use of different biomarkers, phenotypic characteristics of the populations under study and the nutritional strategy used to supplement inorganic NO_3^-. Potentially important is the higher oxidative stress and inflammation in our obese participants which were further increased by the hyperglycaemic insult, and which may have made these participants more responsive to the putative anti-oxidant properties of inorganic NO_3^-. The use of healthy participants in the other two studies (Larsen *et al.*, 2014; Velmurugan *et al.*, 2016) could explain the observed lower responsiveness to the NO_3^- interventions.

4.4.5 *Inorganic NO_3^- and cutaneous microvascular blood flow*

However, the single dose of inorganic NO_3^- used in the present study did not affect microvascular blood flow measured by Laser Doppler. The results are corroborated by the lack of changes in cGMP which suggest that despite the changes in NO_3^-, this has not led to an acute increased activity of the NO signalling. Studies have shown that acetylcholine-mediated dilatation of human skin is unchanged or only partially attenuated following endothelial NO synthase inhibition (Holowatz *et al.*, 2005; Cracowski *et al.*, 2006). Moreover, supplementing type 2 diabetics with beetroot juice for two weeks was ineffective in improving skin microvascular blood flow (Gilchrist *et al.*, 2013). A recently published meta-analysis showed that the effect of inorganic NO_3^- supplementation on endothelial function declined with advanced age (β= -0.01, P = 0.02) (Lara *et al.*, 2015) and this may explain our finding of a significant improvement in endothelial function biomarkers (E- and P-selectin) in younger participants only.

4.4.6 ***Strength and limitations***

In this study, we investigated for the first time, the effects of a single dose of inorganic NO_3^- on hyperglycaemia-induced ED in both younger and older participants. The study had a robust experimental design (randomised, double-blind and cross-over) and to avoid a carry-over effect, we allowed one week wash-out period between each intervention. Previous study demonstrated that the concentrations of NO metabolites return to baseline levels after 24 h of consumption (James *et al.*, 2015).

NO_2^- regarded as the immediate precursor of the active molecule NO (Lundberg *et al.*, 2008). Therefore, measurement of plasma NO_2^- concentration is vital for studies supplementing inorganic NO_3^-. In the present study, blood samples processing was not immediately carried out after collection. Moreover, the solution supposed to stop the reaction of haemoglobin with NO_2^- was not added to the samples. Additionally, the GC-MS method used was not sensitive enough to detect low concentration of NO_2^-. Moreover, we did not use Sievers NO analyser which is regarded as the gold-standard method for measuring NO_2^-/NO_3^- in plasma (Pelletier *et al.*, 2006).

However, previous studies of inorganic NO_3^- supplementation showed a significant rise in plasma NO_2^- concentrations after increased plasma NO_3^- concentrations similar to that observed in this study (Webb *et al.*, 2008; Kapil *et al.*, 2010a). In particular, Cermak et al showed in older overweight and obese diabetic patients (age 66±8.2 y, BMI 29.2±3.9 kg/m^2) similar acute changes over a 2 h period in plasma NO_3^-, which were mirrored by a significant increase in plasma NO_2^- (Cermak *et al.*, 2015).

4.4.7 ***Conclusions***

Overall, our results showed possible positive effects of inorganic NO_3^- supplementation in counteracting the adverse effects of hyperglycaemia on EF. Greater improvement in biomarkers of EF was observed in younger participant. One the other hand, older participants showed greater reduction in oxidative stress and inflammatory markers. These findings may offer novel research questions to be investigated in future studies. Moreover, if confirmed in longer interventions, these findings could have important implications for the prevention and management of pathological processes associated with post-prandial hyperglycaemia.

Chapter 5. Effects of inorganic NO_3^- and vitamin C supplementation on markers of cardiovascular diseases in younger and older healthy participants

5.1 Introduction

Fruits and vegetables consumption is associated with a reduction in cardiovascular mortality and morbidity (Joshipura *et al.*, 2001; Crowe *et al.*, 2011). Several bioactive components of fruits and vegetables are responsible for the cardio-protective effects such as flavonoid, antioxidant vitamins and inorganic NO_3^- (Hooper *et al.*, 2008; Juraschek *et al.*, 2012; Siervo *et al.*, 2013). Antioxidant vitamins such as vitamin C play a major role in conferring beneficial effect by mitigating the earliest stages of atherosclerosis (Aguirre and May, 2008). Vitamin C may ameliorate ED by enhancing NO availability through several mechanisms (Chapter one). However, inconsistent results from large RCTs questioned the protective cardiovascular effects of antioxidant vitamins (Chapter three). Therefore, researchers suggested that inorganic NO_3^- contents have the greatest share of the protective role of fruits and vegetables (Larsen *et al.*, 2006; Lundberg *et al.*, 2006). The NO_3^- contents of green leafy vegetables were found to be an important source of NO_2^- and NO in the circulation (Lundberg *et al.*, 2008; Machha and Schechter, 2012).

Vitamin C and inorganic NO_3^-, individually, proved to have beneficial effects on cardiovascular outcomes (Siervo *et al.*, 2013; Ashor *et al.*, 2014a; Ashor *et al.*, 2015b; Lara *et al.*, 2015) which are related to an enhanced NO production and subsequent improvement of endothelial function (Aguirre and May, 2008; Hobbs *et al.*, 2013). Moreover, we have shown that age might modify the effects of vitamin C and inorganic NO_3^- on cardiovascular markers. While vitamin C effects seemed to be greater in older participants, inorganic NO_3^- effects were larger in younger participants (chapter two and three of this thesis).

The possibility of a mechanistic interaction between vitamin C and NO_3^- and NO_2^- dates back more than 40 years ago, when vitamin C was used to block the conversion of NO_3^- and NO_2^- into *N*-nitroso compounds (NOCs) (Wolff and Wasserman, 1972; Bednar and Kies, 1994). These NOCs were regarded as an important step in the initiation of gastric carcinogenesis (Mowat *et al.*, 1999). The purported mechanisms of the beneficial effects of vitamin C included the wash-out of NO_2^- by converting it into NO and, therefore, preventing the formation of NOCs (Mowat *et al.*, 1999).

Several studies reported the possibility of interaction of vitamin C with NO_3^--NO_2^--NO pathway (Scorza *et al.*, 1997; Sibmooh *et al.*, 2008; Garcia-Saura *et al.*, 2012). Vitamin C may enhance the conversion of NO_2^- into NO in the circulation, therefore, further contributing to the NO pool (Lundberg *et al.*, 2008). Besides the circulatory interaction, studies have shown the possibility of NO_2^--vitamin C interaction in the oral cavity (Duncan *et al.*, 1995; Takahama *et al.*, 2008), stomach (Mowat and McColl, 2001) and kidney tubules (Carlsson *et al.*, 2001).

In the present study, we hypothesized that inorganic NO_3^- and vitamin C co-administration will have greater increase in NO availability than each intervention alone. Therefore, greater improvement in BP, skin microvascular blood flow and arterial stiffness would be expected from the anticipated synergistic interaction. Synergism is the situation in which the co-administration of two agents will produce greater effect than the algebraic sum of their independent actions when given separately (Tallarida, 2011). Furthermore, we hypothesized that the effects of inorganic NO_3^- and vitamin C would vary in younger and older participants.

In a 2×2 crossover RCT, we aimed to investigate the potential, additive or synergistic effects of vitamin C and inorganic NO_3^- administration on resting BP, heart rate variability (HRV) and vascular function. The secondary aim was to investigate whether age modifies putative interactions between vitamin C and inorganic NO_3^- on these outcomes. To understand some of the mechanisms of this potential interaction we measured circulatory cardiovascular markers such as NO metabolites (NO_3^- and NO_2^-), cGMP, tetrahydrobiopterin (BH4), dehydroascorbic acid (DHA) and 3 nitrotyrosine (3- NT).

5.2 Methods

5.2.1 *Participants*

The study included twenty volunteers (9 males, 11 females) with a mean age of 45.75±1.9 years (range: 18-70 years) and a mean body mass index (BMI) of 24.3±0.8 kg/m^2 (range: 20-29.9 kg/m^2). Participants were recruited from members of staff or the general public who responded to our newspaper advertisement or emails targeted towards potential volunteers. All the study visits were conducted in the Clinical Ageing Research Unit (CARU), Newcastle University from November 2014 to November 2015. The trial was performed in accordance with the Declaration of Helsinki and the principles of the International Conference on Harmonization-Good Clinical Practice (ICH-GCP) guidelines. The study was approved by the regional ethics committee of East of Scotland Research Ethics Service (EoSRES) (REC reference:

14/ES/0059, Appendix D) and was registered with Current Controlled Trials (ISRCTN98942199).

5.2.2 *Participants screening and exclusion criteria*

Participants who approached the research team with the intention to participate in the study received detailed information about the study. Potential eligible volunteers were then contacted via telephone to ensure an accurate evaluation of the inclusion and exclusion criteria including socio-demographic status, smoking history and confirming the participants' commitment and availability to complete the study visits. If participants passed this first initial screening phase, they were then invited to CARU for a short screening visit to confirm their eligibility for the study.

Participants were excluded if they had been diagnosed with medical conditions (i.e. diabetes, cancer or systemic inflammatory disorders) or had blood pressure of ≥ 180 mmHg systolic or ≥ 110 mmHg diastolic at the screening visit. Participants were also excluded if they reported taking medications or nutritional supplements (i.e. organic nitrates, corticosteroids, diuretics, hormonal therapies or multivitamins) that could interfere with the nutritional interventions (inorganic NO_3^- and vitamin C) or study outcomes. In addition, individuals were excluded if they were smokers, vegetarian, reported alcohol intake greater than 21 U/week for men and 14 U/week for women, or their body weight had changed more than 3 kg within the last 2 months. Participants who reported history of major surgeries or blood donations in the last 3 months were also excluded from the study.

5.2.3 *Study design and randomisation*

This was a Latin-square 2×2 factorial cross-over randomised, double-blind, placebo controlled study. The randomisation was conducted by an independent investigator outside the research team. The preparation and labelling of the solutions (inorganic NO_3^-, vitamin C and their placebos) were performed by the Pharmacy Department of the Newcastle upon Tyne Hospitals (Royal Victoria Infirmary). The safety and accessibility of the randomisation codes was guaranteed by a member of the CARU Research Facility outside the research team. Interventions (inorganic NO_3^- or vitamin C) and the corresponding placebos solutions had the same characteristics and had identical volume, colour and presentation.

5.2.4 ***Sample size calculation***

Prior to this study, the combined effects of inorganic nitrate and vitamin C on cardio-metabolic outcomes had not been tested in humans and therefore experimental information was not available to inform our sample size calculations. In addition the influence of ageing on the effects of these nutritional interventions has never been assessed. Therefore we based our calculations on the differences that would be detected in each age group after the combined interventions. Our calculations are based on a minimal difference of ±3mmHg in SBP (SD = ±3mmHg) between placebo and combined interventions. Using a matched paired design, a sample size of 10 subjects for each age group was required to detect a significant difference in SBP with a power of 80% and alpha of 0.05. Therefore, we recruited a total of 20 participants (10 younger and 10 older) who received all the 4 interventions in a crossover design. The sample size calculation was performed using G*Power 3.1 (Heinrich Heine Universität Düsseldorf, Germany).

5.2.5 ***Nutritional supplements***

The active NO_3^- supplementation contained 7 mg [0.1 mmol/kg] of potassium NO_3^- per kg of body weight in sterile water while its placebo was consisted of a matched amount of potassium chloride (7 mg per kg of body weight). The active vitamin C supplementation contained 20 mg ascorbic acid per kg body weight and its placebo consisted of matched volume of Sodium chloride 0.9%. Participants were randomised to the following interventions: 1) NO_3^- supplementation (active) + vitamin C (active); 2) NO_3^- supplementation (active) + vitamin C (placebo); 3) NO_3^- supplementation (placebo) + vitamin C (active); 4) NO_3^- supplementation (placebo) + vitamin C (placebo). Each participant received all four interventions in four consecutive visits. As mentioned above, each visit was separated by a 7-day washout period. The NO_3^- dose corresponded to the amount normally found in 150–250 g of a NO_3^--rich vegetable such as spinach, lettuce or beetroot (Larsen *et al.*, 2007). The dose of vitamin C implemented in this study was 20 mg/kg which means 1400 mg in a 70 kg adult person. This dose based on our previous observation that the lowest effective dose to improve EF was around 1000 mg per day (Ashor *et al.*, 2014a).

5.2.6 ***Study protocol***

a. *Screening visit:* Eligible subjects, from the initial telephone interview, were invited to the research unit in fasting conditions (at least 12 hours). At the screening visit, potential volunteers were given detailed information of the study and their BMI and blood pressure

were checked. Eligible subjects were, then, asked to complete a written consent to participate in the study. Additionally, a blood sample obtained for the assessment of plasma NO_3^- and vitamin C concentrations. At the end of that visit, participants were asked to follow a run-in diet (low NO_3^-/low vitamin C diet, Appendix E) and to avoid using antibacterial mouthwash and chewing-gum for two days prior to each study visit. The run-in diet ensured a standardized NO_3^- and vitamin C intake among study participants. The mouthwash and chewing-gum restriction avoided any interference with the salivary conversion of NO_3^- into NO.

b. *Study visits (1, 2, 3 and 4):* Participants arrived early in the morning (~8.00am) in fasting condition (~ 12 hours). Participants were then randomised to one of the four interventions (inorganic NO_3^-, vitamin C, both, or placebo). Body weight, height, waist circumference were measured and body composition (fat mass, fat free mass) assessed by using bioelectrical impedance analysis (Tanita BC420 MA, Tanita Corporation, Tokyo, Japan). Heart rate variability (HRV) was monitored throughout the study visit by fitting a strap around the participants' chest and which was remotely connected to a wrist watch (Polar S810, Polar Electro). A cannula was then inserted in the ante-cubital vein while subjects were lying in a supine position. Blood pressure (systolic, diastolic) was measured at baseline (pre-dose administration) and then every 30 minutes after the administration of the dose. Endothelial function was assessed by post-reactive occlusion hyperaemia and pulse wave velocity at baseline and 180 min post-interventions. Blood samples were collected at 0, 30, 60, 120 and 180 minutes. Participants were asked to complete a food frequency questionnaire to assess their typical food intake. Physical activity levels and dietary nitrate intake (Appendix B) over the week prior to each visit were documented by completing two short questionnaires. After each study session, the cannula was removed from the participants' arm and a small breakfast was offered to the participant. Participants were asked to return to their normal eating habits during the wash-out period and to follow the low nitrate/low vitamin C run-in diet for 2 days prior to their next study visit. This protocol was repeated at the second, third and fourth (last) visit and each time the nutritional interventions were administered as dictated by the randomisation order. A detailed description of the study phases and measurement protocols conducted during the study is provided in Figure 5.1.

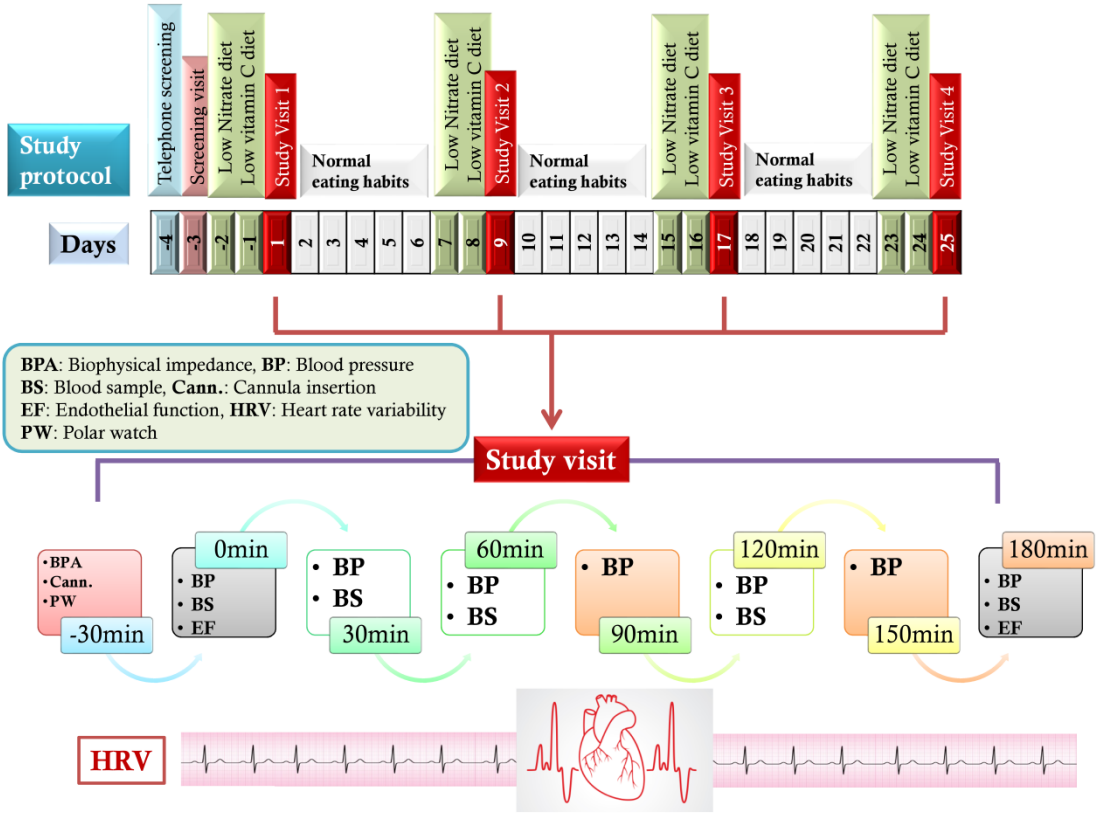


Figure 5.1: Study protocol and description of succession of measurements conducted at each study visit

5.2.7 *Blood pressure*

Baseline BP was measured five times at 1-minute intervals using a semi-automated blood pressure recorder (Dinamap V100; GE Medical Systems, Milwaukee, WI, USA). The mean of the five records was taken as the baseline values of blood pressure. Subsequently, BP was measured at 30, 60, 90, 120, 150, 180 minutes from the administration of the interventions. Measurements of BP were conducted in a rested, supine position and results were concealed from each participant.

5.2.8 *Cutaneous microvascular blood flow*

Cutaneous microvascular reactivity was measured during post-occlusive reactive hyperaemia (PORH) of the forearm using laser Doppler (Moor LDF, Moor Instruments, Axminster, UK). All assessments were performed in a temperature-controlled room (22-24 °C) following an acclimatisation period of 15 minutes. With the subject supine, the laser Doppler probe was attached to the right forearm. Resting cutaneous blood flow was recorded for 5 minutes followed by a 3-minute inflation of right upper-arm pressure cuff to 200 mmHg to occlude

brachial artery. The hyperaemic response was recorded for 4 minutes following the deflation of the pressure cuff. The mean blood flow of the selected area was determined with the help of the Moor VMS V3.1 software and expressed in perfusion units (PU) in relation to an internal standard calibration of the device. The PORH index was calculated as the ratio of the area under curve (AUC) one minute after the release of the cuff relative to the AUC of a one minute period before cuff inflation (Figure 5.2).

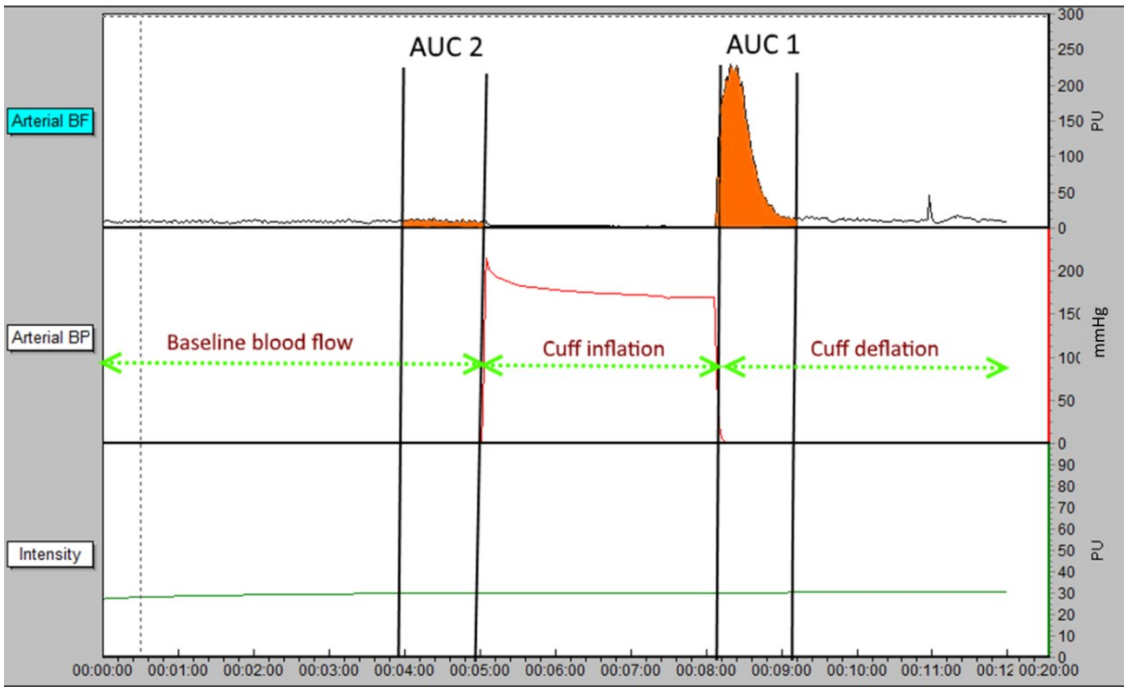


Figure 5.2: Example of Laser Doppler blood flow recording of study participant. The protocol lasted for 12 minutes (5 minutes resting blood flow were recorded, 3 minutes were pressure cuff around the arm inflated to 200mmHg and the last 4 minutes of recording were after the release of pressure cuff). AUC: area under curve; BP: blood pressure; PU: perfusion unit.

5.2.9 *Pulse wave velocity*

Photoplethysmography (PPG), developed by the Medical Physics Department of Newcastle University, was used to measure pulse wave velocity (PWV) and the distensibility coefficient (DC) (Figure 5.3). The PPG probes, which included a matched infrared emitter, were used to quantify changes in blood volume in the index finger and earlobe. In addition, a three-electrode ECG signal was also recorded to provide a timing reference for the PPG pulses. The signals from each of the PPG probes and ECG electrodes were fed to a data capture computer (Fujitsu Lifebook S3020 laptop, Japan). Special software, named ARD (Analogue Recorder and

Display) which had been developed in the Medical Physics Department was used for the PPG and ECG signal acquisition, display and data storage.

All assessments were performed in a temperature-controlled room (22-24 °C) following a resting period of 15 minutes. PPG probes were attached to the same side index fingertip and earlobe. Right, left arms and left leg ECG probes were also attached to the participant's body. Once good signals from PPG probes and ECG were obtained on the computer, recording started and continued for 120 seconds (Figure 5.4). The distance between the fingertip to the sternal notch and fingertip to the earlobe was measured. This distance used to calculate the propagation velocity of the pulse wave across the arterial wall. DC were derived from PWV using a formula described by Zheng and Murray (2011).

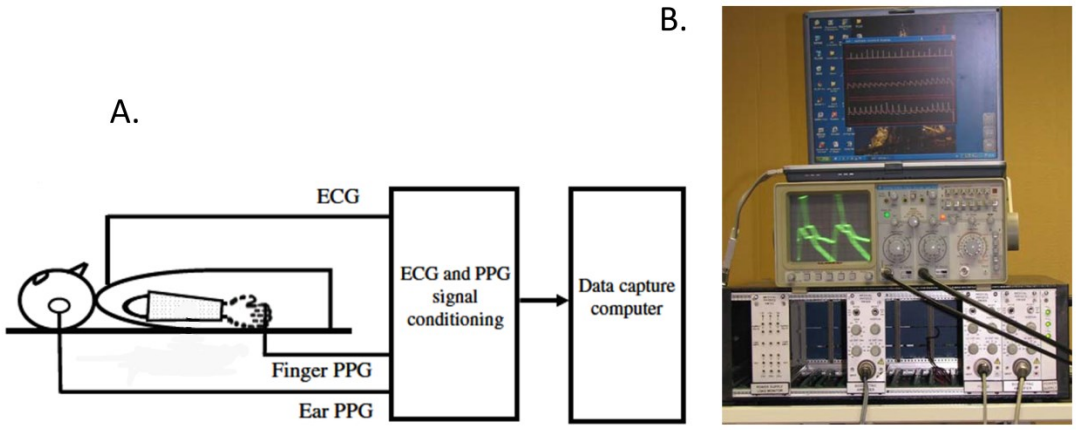


Figure 5.3: Photoplethysmography (PPG). A) Schematic diagram of the measurement protocol of pulse wave velocity; B) The data capture computer (Fujitsu Lifebook S3020 laptop, Japan) and the ARD (Analogue Recorder and Display) software. Adapted from Zheng and Murray (2011)

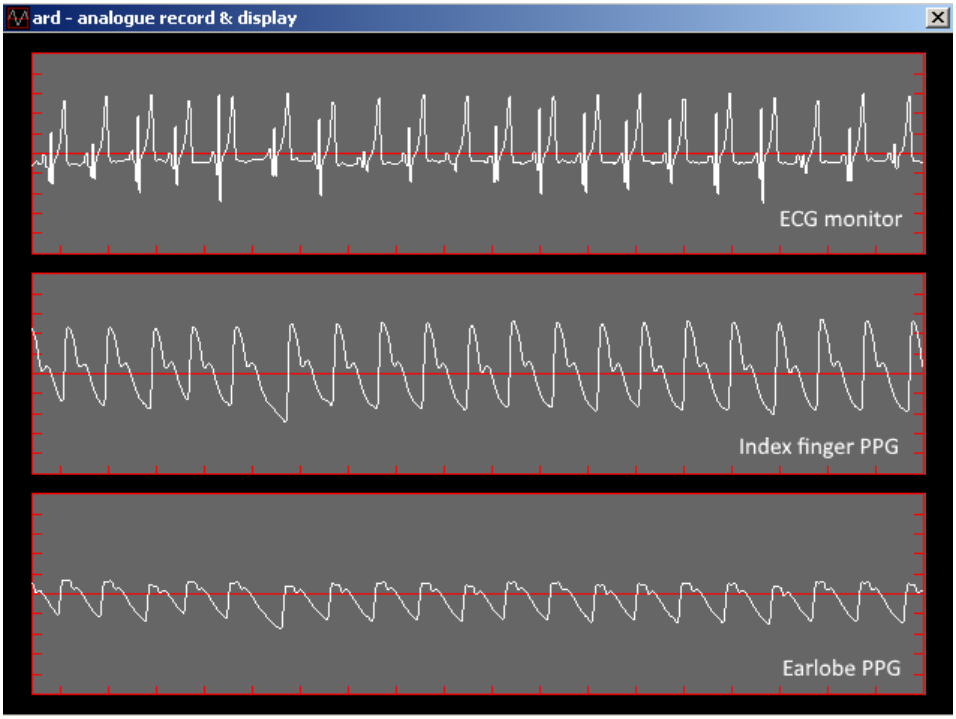


Figure 5.4: Example of pulse wave recording of study participant. PPG: photoplethysmography

5.2.10 ***Heart rate variability (HRV)***

A portable, automated device was used to assess components of HRV and to provide information on autonomic balance (RS800; Polar Electro, Kemple, Finland). The chest strap of the Polar RS800 was fixed around the participant's chest according to the manufacturer's instructions. The interbeat interval (R–R series) of the Polar equipment was stored automatically in the watch and later transferred to a laptop computer. The R–R series data were analysed using Protrainer Polar 5 software (version 5.40.171, Polar Electro).

The following indices of HRV were extracted: 1) RMSSD (milliseconds): square root of the mean of the squared differences of successive normal R-R intervals over a full 3hr recording. This measurement reflects the beat-to-beat variance in HR mediated principally by parasympathetic activity (Vanderlei *et al.*, 2009); 2) pNN50 (%): the percentage of intervals that differ from each other by more than 50ms. This is correlated with parasympathetic activity (Vanderlei *et al.*, 2009); 3) low frequency/high frequency ratio measured in milliseconds (LF/HF) which is an indirect measure of autonomic balance (sympathetic/parasympathetic balance) (Vanderlei *et al.*, 2009); 4) SD1 (milliseconds): is an indicator of the standard deviation of the instantaneous RR variability and is considered a parasympathetic index of sinus node control (Hoshi *et al.*, 2013); 5) SD2 (milliseconds): is an indicator of the standard

deviation of the continuous or long-term variability of the heart rate. SD2 is influenced by both parasympathetic and sympathetic tones (Hoshi *et al.*, 2013).

5.2.11 *Blood sample processing and biochemical assays*

At each predetermined time point, blood samples were collected in 4 tubes (4mL each). Two tubes contained lithium heparin and one tube each contained EDTA (Ethylenediaminetetraacetic acid) and serum gel. Blood samples were processed immediately after collection. Stop solution was added to aliquots intended for measuring NO metabolites (0.25mL of stop solution added to 1mL of plasma).

The purpose of stop solution is to stop the reaction of nitrite with oxy- and deoxyhaemoglobins (Hb) by oxidising the heme to form metHb. The stop solution consists of potassium ferricyanide (MW 329.24), N-ethylmaleimide (MW 125.12) and Nonidet P-40 (all from Sigma-Aldrich) (Pelletier *et al.*, 2006). A new stock of stop solution was prepared fresh in every study visit day. 1320 mg of potassium ferricyanide and 65 mg of N-ethylmaleimide were added to 4.5 mL of distilled water and mixed them thoroughly to remove any remaining particles. Then, 500 µL of Nonidet P-40 were added and the solution was mixed gently to avoid excessive foaming (Pelletier *et al.*, 2006).

Blood samples were spun at 5000x g for 5 minutes at 4°C and serum and plasma aliquots were stored immediately at -80°C until further analyses. These samples were then used to measure NO_3^-, NO_2^-, vitamin C, dehydroascorbic acid (DHA) cyclic guanosine monophosphate (cGMP), tetrahydrobiopterin (BH4) and 3-nitrotyrosine (3-NT) concentrations.

5.2.12 NO_3^- *and* NO_2^- *analyses*

Plasma concentrations of NO_3^- and NO_2^- were analysed at Professor Alan Schechter's laboratory of biomolecular medicine at the National Institutes of Health (NIH), Bethesda, USA using a Sievers gas-phase chemiluminescence nitric oxide 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 (I_3) 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 concentrations of NO_3^- and NO_2^- standards. To analyse data from the injected samples, two types of software were used: data acquisition and analysis software. For data acquisition the Liquid Program (version 3.21) was used and for data analysis the Origin software (version 10; OriginLab) was then applied to obtain the AUC (measured in mV). A detailed description of the protocol is provided in (Appendix F).

5.2.13 *Vitamin C analysis*

Vitamin C concentration in plasma samples was estimated using ESA CoulArray high performance liquid chromatography (HPLC). All samples were injected from an autosampler at 0-2 ºC. The column was Hypersil GOLD AX (diameter: 4.6mm; length: 250mm; particle size: 5µm; Thermo Fisher Scientific). The flow rate was 1.2mL/minute and the injection volume 20µL. The mobile phase was Ammonium Acetate (100mM, pH 6.8) and acetonitrile (30:70). 10% meta-phosphoric acid (MPA) was used to stabilise vitamin C in plasma samples.

5.2.14 *DHA analysis*

DHA is the oxidised form of vitamin C. DHA is transported intracellularly (e.g. erythrocytes) were it is regenerated to ascorbic acid by several intracellular mechanisms (Lykkesfeldt, 2007). Increased tissue DHA regarded as a sign of redox imbalance and inadequate recycling capacity (Lykkesfeldt, 2007). DHA Microplate Assay Kits (Cohesion Biosciences) were used to analyse DHA in our plasma samples. The principle of the assay is initiation of enzymatic catalysis of the DHA by dithiothreitol (DTT). The enzyme reaction product (ascorbic acid) then can be measured with colorimetric plate reader at 265nm.

5.2.15 *cGMP analysis*

Assay principles of cGMP analysis were described in section 4.2.8 of chapter four of this thesis.

5.2.16 *BH4 analysis*

Vitamin C stabilizes, and increases the synthesis, of BH4, a cofactor which is essential for the function of eNOS. BH4 deficiency leads to eNOS uncoupling and the production of superoxide instead of NO (Schmidt and Alp, 2007). We analysed plasma concentration of BH4 using Human Tetrahydrobiopterin Elisa Kit (MBS283103, MyBiosource, Inc. San Diego, USA). This is a competitive enzyme immunoassay technique.

5.2.17 *3-NT analysis*

3-NT considered as an index of oxidative stress (Ischiropoulos, 2009). It is formed by the interaction of reactive nitrogen species (peroxynitrite anion, nitrogen dioxide) with the tyrosine residues of proteins (Radi, 2004). This reaction considered as a post-translational modification of these proteins with consequent changes in its function (Ischiropoulos, 2009). Moreover, 3-NT concentrations strongly correlated with CVD (Shishehbor *et al.*, 2003). Significantly higher levels of NT were found among patients with CAD (Shishehbor *et al.*, 2003). I used ELISA kits (ab210603, Abcam, Cambridge, USA) to analyse 3-NT in serum samples. This ELISA kits utilizes nitrotyrosine-coated plates and horseradish peroxidase (HRP)-conjugated antibodies to detect 3-NT in the samples.

5.2.18 *Statistical analysis*

Data were checked for normality and appropriate transformations were applied for non-normally distributed data. Summary data are presented as means ± SEM. For outcomes with repeated measurements over time (BP and plasma concentrations of vitamin C, DHA, NO_3^-, NO_2^-, cGMP, BH4 and 3-NT), we calculated the mean values of the multiple measurements post-intervention with an adjustment for baseline in the model and the area under curve (AUC) over 3 hrs using the trapezoid method (6.11Appendix G). The mean differences were calculated for EF outcomes (PORH index, PWV and DC) by subtracting the baseline measurements from the measurements at the end of the visits. Figure 5.5 illustrates the process followed in the statistical analysis of this study. Linear mixed models were applied to analyse the data using Stata version 13 (StataCorp LP, TX, USA).

The statistical model examined the effects of each dietary agent (inorganic NO_3^- or vitamin C) individually and for a potential interaction effect (interaction between inorganic NO_3^- and vitamin C). In addition, the possibility of age interaction examined by including age as fixed factor in the statistical model. The main effects of inorganic NO_3^- investigated by comparing groups who were given NO_3^- with groups who were not (Nitrate + vs. Nitrate -). The main effects of vitamin C studies by comparing groups who were given vitamin C with groups who were not (Vitamin C + vs. Vitamin C -). Fixed factors include inorganic NO_3^-, vitamin C (within-subject factors) and age (between-subject factor). Subjects' identification was regarded as random factor. Due to the presence of significant interaction between age, inorganic NO_3^- and vitamin C, statistical analyses per group (inorganic NO_3^-, vitamin C and the combination of both interventions versus placebo) were presented. GraphPad prism version 7 (GraphPad

software, CA, USA) was used to construct the graphs. *P* values < 0.05 were considered to indicate statistical significance.

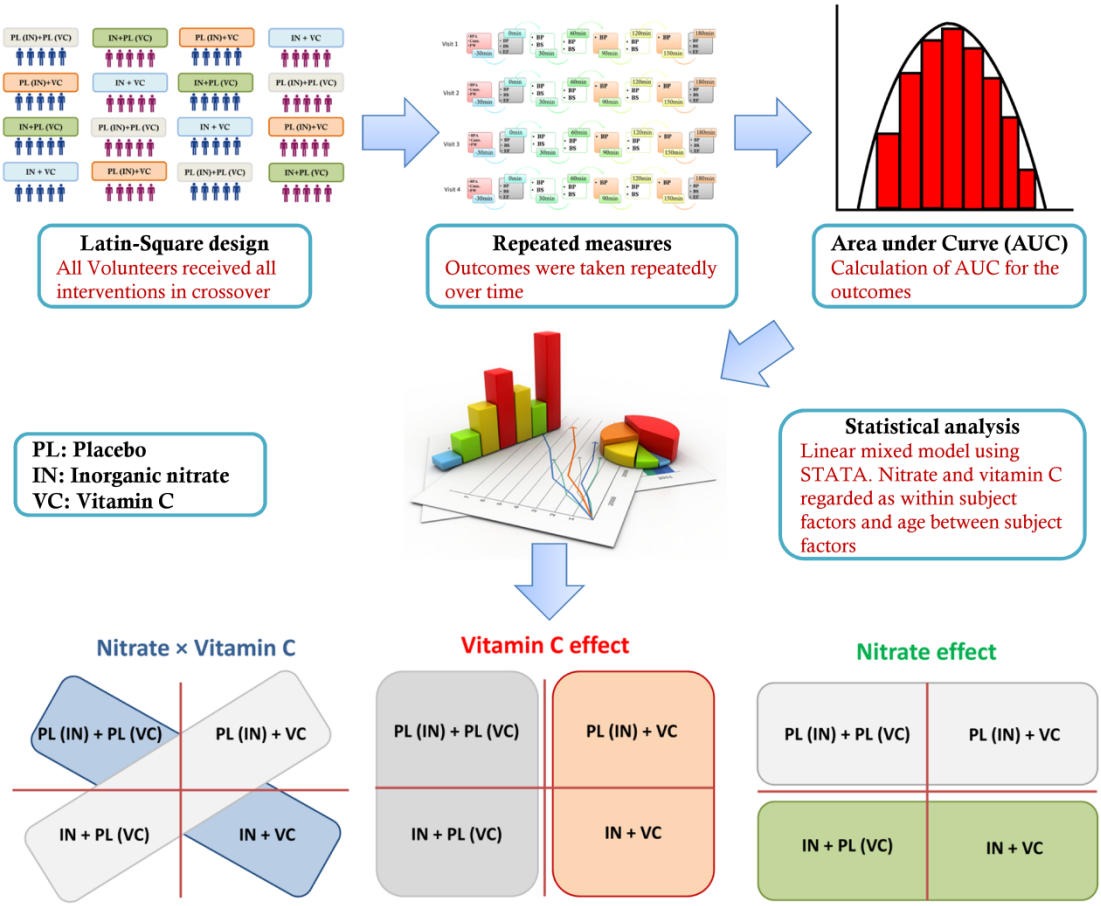


Figure 5.5: The statistical plan applied to analyse the study results

5.3 Results

5.3.1 *Recruitment and baseline characteristics of the participants*

A total of 20 participants were randomised to the interventions, ten younger (29.2±0.8 years) and ten older (62.3±0.6 years) participants (Figure 5.6). All participants completed all the four study visits. The interventions were well-tolerated and no adverse effects were reported. Baseline characteristics of the participants (Table 5.1) show that the younger and older participants were well-matched for anthropometric variables, dietary energy intake and vitamin C intake. However, BP was significantly higher in older participants ($P < 0.01$).

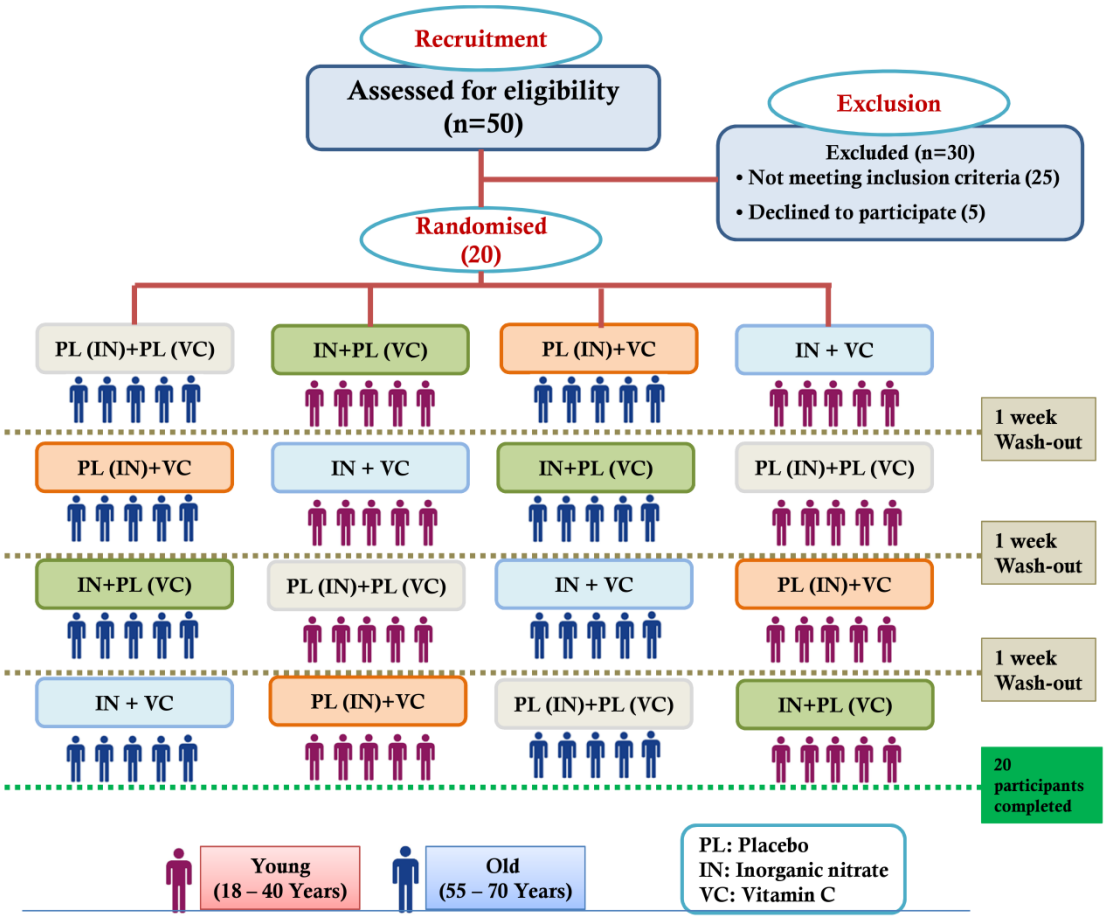


Figure 5.6: Flow diagram of the study

Table 5.1: Baseline characteristics of younger and older participants[1]

	All	Younger	Older	*P* value
Number	20	10	10	
Gender (male/female)	9/11	5/5	4/6	
Age (years)	45.8±3.9	29.2±1.7	62.3±1.3	0.0001
Body mass index (kg/m^2)	24.3±0.6	24.5±0.7	24.2±1	0.82
Waist circumference (cm)	84.9±2.1	84.8±2.4	85.2±3.7	0.94
Fat mass (kg)	18.6±1.1	18.4±1.4	18.9±1.6	0.83
Systolic blood pressure (mmHg)	115.7±3.9	106 ±2.6	125±6	0.009
Diastolic blood pressure (mmHg)	66.3±2	60.5±1.4	72±2.7	0.001
Mean arterial pressure (mmHg)	85.2±2.7	77.6±1.6	92.8±3.8	0.002
Energy intake (KJ/day)	12204±983	13638±1720	10769±815	0.14
Vitamin C intake (mg/day)	170.5±20	184.5±28	156.5±28	0.48
Nitrate intake (mg/day)	176.51±11.6	179.1±20	173.9±12	0.82
Physical activity, METs/wk[2]	3440±322	3142±390	3801±533	0.31
Plasma nitrate (μmol/L)	27.3±2	29.6±2.8	25.1±2.7	0.27
Plasma nitrite (nmol/L)	238±24	245±43	231±23	0.78
Plasma vitamin C (μmol/L)	56.9±4	51±7	62.4±3	0.17
Plasma BH4[3] (pg/mL)	565±60	464±64	665±92	0.10
Serum 3-nitrotyrosine (nmol/mL)	592±60	543±87	640±86	0.43

[1]Data shown as mean±SEM. [2]MET: Metabolic Equivalent of Task, [3]BH4: tetrahydrobiopterin

5.3.2 NO_3^- and NO_2^- concentrations

Compared with placebo, plasma NO_3^- was significantly increased after inorganic NO_3^- (younger: Δ 177 μmol/L, 95% CI: 160, 195; older: Δ 177 μmol/L, 95% CI: 160, 195) and inorganic NO_3^- + vitamin C intake (younger: Δ 173 μmol/L, 95% CI: 155, 191; older: Δ 196 μmol/L, 95% CI: 178, 213) (Figure 5.7A). Similarly, relative to control, plasma NO_2^- was significantly increased after inorganic NO_3^- (younger: Δ 135 nmol/L, 95% CI: 76, 194; older: Δ 206 nmol/L, 95% CI: 147, 265) and inorganic NO_3^- + vitamin C intake (younger: Δ 125 nmol/L, 95% CI: 66, 184; older: Δ 184 nmol/L, 95% CI: 125, 243) (Figure 5.7B). However, no significant differences in plasma NO_3^- and NO_2^- were observed after vitamin C intake (Figure 5.7). Moreover, no significant difference was reported between younger and older participants. Vitamin C did not significantly modify the concertation of plasma NO_3^- and NO_2^- when co-administered with inorganic NO_3^-.

5.3.3 *Vitamin C and DHA concentrations*

In comparison with placebo, vitamin C concentration significantly increased after vitamin C (younger: Δ 41 μmol/L, 95% CI: 32, 51; older: Δ 45 μmol/L, 95% CI: 35, 54) and inorganic NO_3^- + vitamin C intake (younger: Δ 39 μmol/L, 95% CI: 29, 48; older: Δ 52 μmol/L, 95% CI: 42, 61) (Figure 5.8A). However, only older participants demonstrated a significant increase in DHA concentration after vitamin C supplementation (Δ 0.8 μmol/mL, 95% CI: 0.01, 1.6) (Figure 5.8B). Additionally, no significant differences were seen after inorganic NO_3^- supplementation. Lastly, no interactions between age, inorganic NO_3^- and vitamin C were reported.

5.3.4 *Systolic, diastolic and mean BP*

Overall, systolic, diastolic and mean arterial BP were significantly higher in older than younger participants (systolic: Δ 15.6 mmHg, 95% CI: 7.1, 24; diastolic: Δ 8.9 mmHg, 95% CI: 4.3, 13.4; mean BP: Δ 12 mmHg, 95% CI: 6.5, 17.4) (Figure 5.9A, Figure 5.10A and Figure 5.11A). In older participants, systolic BP was significantly reduced after inorganic NO_3^- (Δ -3.7 mmHg, 95% CI: -6.8, -0.6) and vitamin C (Δ -3.9 mmHg, 95% CI: -6.9, -0.8) intake (Figure 5.9A). Likewise, single and combined supplementation of inorganic NO_3^- and vitamin C significantly reduced mean BP in older participants only (vitamin C: Δ -2.4 mmHg, 95% CI: -4.7, -0.11;

inorganic NO_3^-: Δ -2.7 mmHg, 95% CI: -5.0, -0.43; inorganic NO_3^- + vitamin C: Δ -2.6 mmHg, 95% CI: -4.9, -0.27) (Figure 5.11A). Moreover, age significantly modified the effects of vitamin C on systolic, diastolic and mean arterial BP ($P < 0.05$) (Figure 5.9B, Figure 5.10B and Figure 5.11B). Younger participants demonstrated a significant increase in diastolic BP after vitamin C supplementation (Δ 2.2 mmHg, 95% CI: 0.02, 4.4) (Figure 5.10A). Additionally, marginally significant interaction was found between inorganic NO_3^- and vitamin C for diastolic BP ($P = 0.05$) (Figure 5.10B).

5.3.5 ***Physiological markers of EF***

Inorganic NO_3^- supplementation marginally improved PORH index in both younger and older participants (younger: Δ 2.2 AU, 95% CI: -0.003, 4.3 [$P = 0.05$]; older: Δ 2.1 AU, 95% CI: -0.11, 4.2 [$P = 0.06$]) (Figure 5.12A). However, in older participants, inorganic NO_3^- + vitamin C intake produced greater improvement in arterial stiffness indices (PWV: Δ -2 m/s, 95% CI: -3.61, -0.32; DC: Δ 0.03 % per mmHg, 95% CI: 0.002, 0.06) than the sum of the effects of inorganic NO_3^- and vitamin C supplemented individually (Figure 5.12B and Figure 5.12C).

5.3.6 ***Indices of HRV***

HRV indices tend to be lower in older than younger participants (RMSSD: Δ -22 ms, 95% CI: -45, 1.4; pNN50: Δ -9.4 %, 95% CI: -15, -3.7) (Figure 5.13 and Figure 5.14). Nevertheless, inorganic NO_3^-+ vitamin C intervention significantly improved RMSSD and pNN50 in older participants (RMSSD: Δ 21 ms, 95% CI: 6.8, 35.8; pNN50: Δ 3 %, 95% CI: 0.16, 5.7). Moreover, the effects of inorganic NO_3^-+ vitamin C on RMSSD and pNN50 was greater than the effects of both intervention supplemented separately (Figure 5.13 and Figure 5.14).

Likewise, intervention with inorganic NO_3^- significantly improved SD1 and SD2 indices in older participants (SD1: Δ 56.2 ms, 95% CI: 17.6, 94.8; SD2: Δ 323 ms, 95% CI: 98, 548) (Figure 5.15 and Figure 5.16). In contrast, vitamin C intake significantly reduced pNN50 in younger participants (Δ -3.3 ms, 95% CI: -5.9, -0.57) (Figure 5.14B). However, there was a tendency of beneficial interaction of inorganic $NO_3^-$and vitamin C on pNN50 ($P = 0.05$) (Figure 5.14C). No changes in LF/HF ratio were reported following all interventions (Figure 5.17).

5.3.7 ***Circulatory biomarkers***

Relative to placebo, vitamin C supplementation marginally increased cGMP concentration in older participants (Δ 5.4 pmol/mL, 95% CI: -0.46, 11.3) (Figure 5.18A). Moreover, inorganic + vitamin C significantly increased BH4 concentration in the same age group (Δ 145 pg/mL, 95% CI: 41.3, 250) (Figure 5.18B). In contrast, inorganic + vitamin C significantly reduced 3-NT in younger participants only (Δ -93.5 nmol/mL, 95% CI: -170, -17.4) (Figure 5.18C). The effect of inorganic + vitamin C co-administration on BH4 and 3-NT concentrations was greater than the sum of the effects of both intervention given separately (Figure 5.18B and Figure 5.18C).

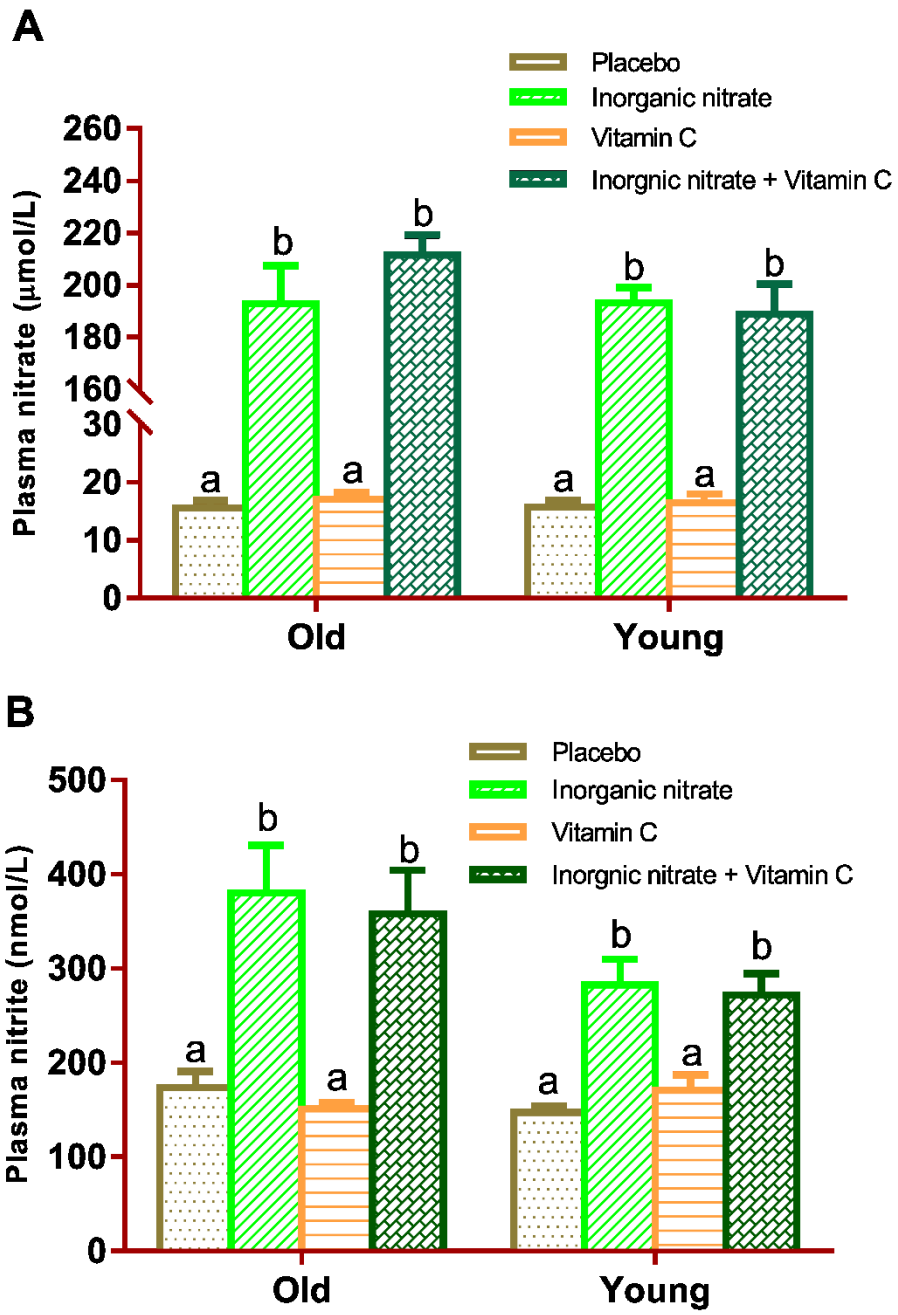


Figure 5.7: Plasma NO_3^- (A) and NO_2^- (B) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05).

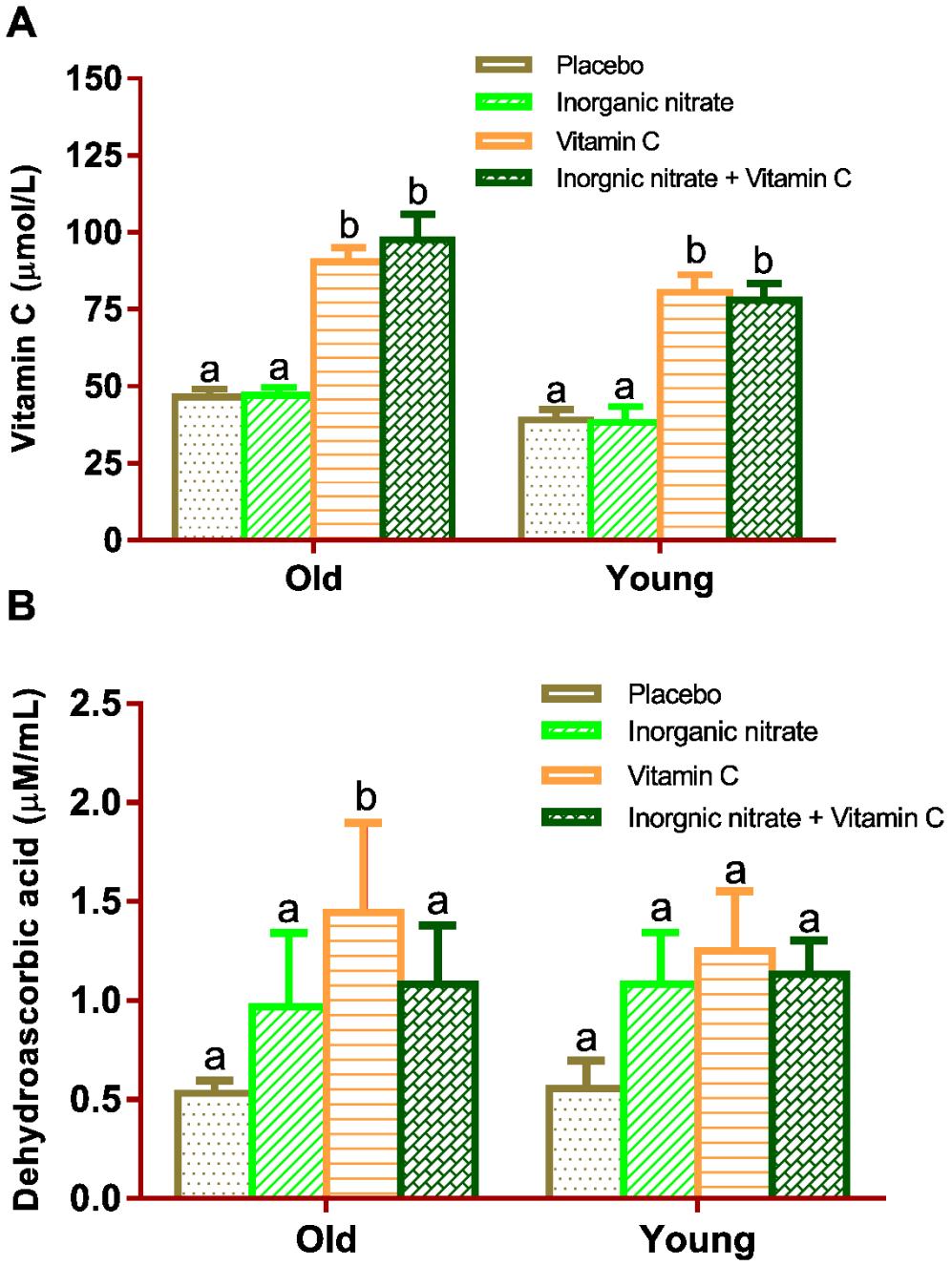


Figure 5.8: Plasma vitamin C (A) and dehydroascorbic acid (DHA) (B) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). *: P < 0.05.

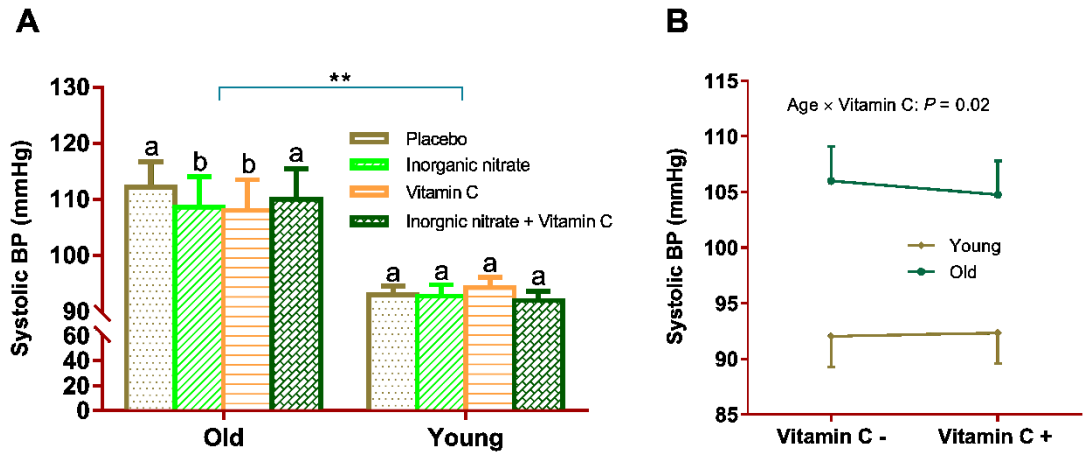


Figure 5.9: Systolic blood pressure (BP) (A) and the age × vitamin C interaction (B) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). **: P < 0.01.

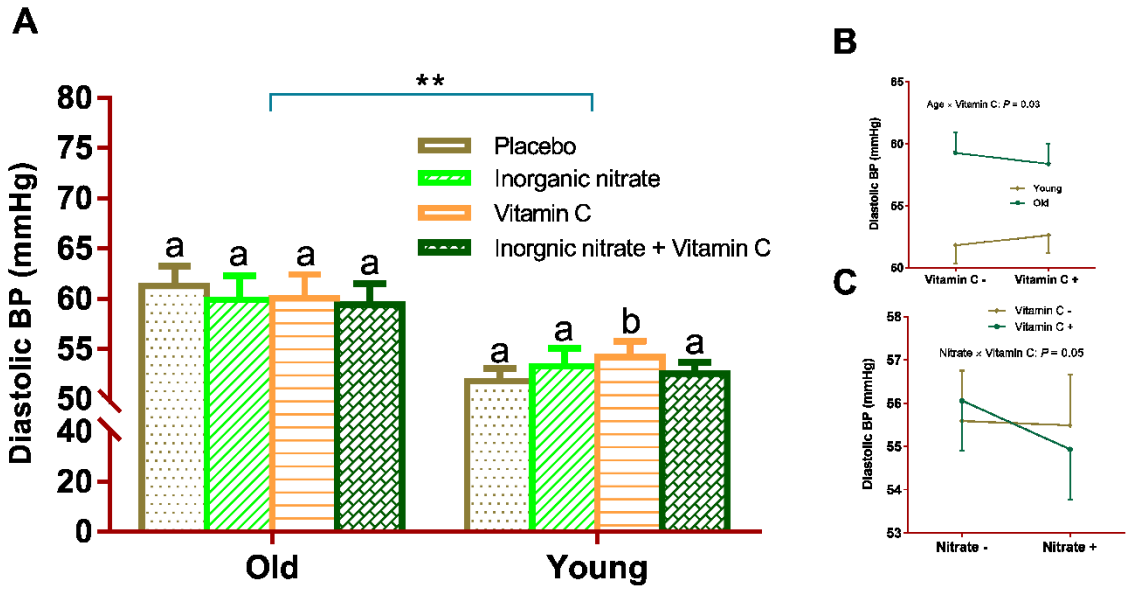


Figure 5.10: Diastolic blood pressure (BP) (A), the age × vitamin C interaction (B) and inorganic NO_3^- × vitamin C interaction (C) in younger (*n*= 10) and older participants (*n*= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). **: P < 0.01.

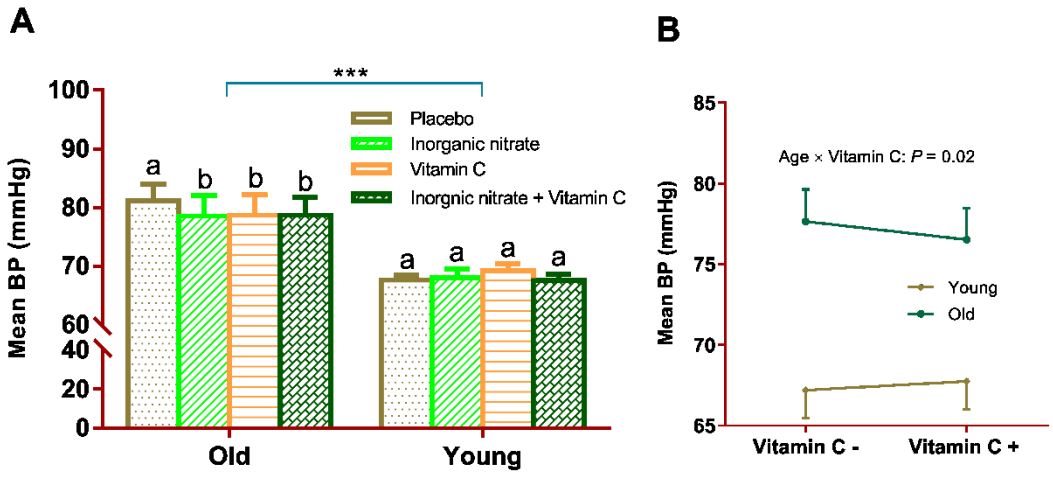


Figure 5.11: Mean arterial blood pressure (BP) (A), the age × vitamin C interaction (B) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). ***: P < 0.001.

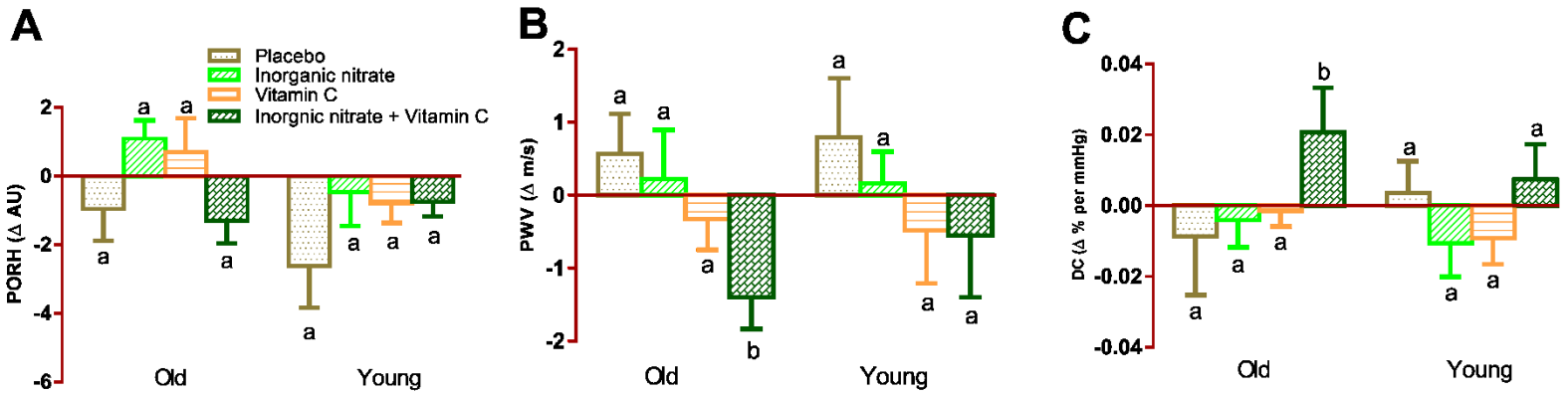


Figure 5.12: Post-occlusive reactive hyperaemic blood flow (PORH) index (A) Pulse wave velocity (PWV) (B) and the distensibility coefficient (DC) (C) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). m/s: meter per second.

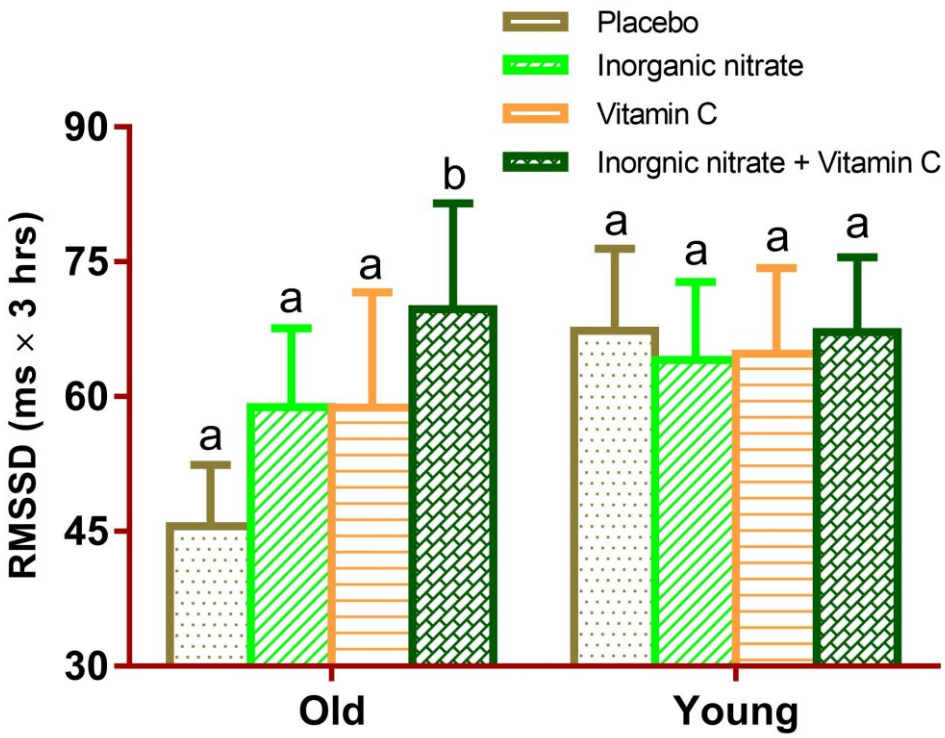


Figure 5.13: Square root of the mean of the squared differences (RMSSD) of successive normal R-R intervals over a full 3 hours recording in younger (*n*= 10) and older participants (*n*= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). ms: millisecond.

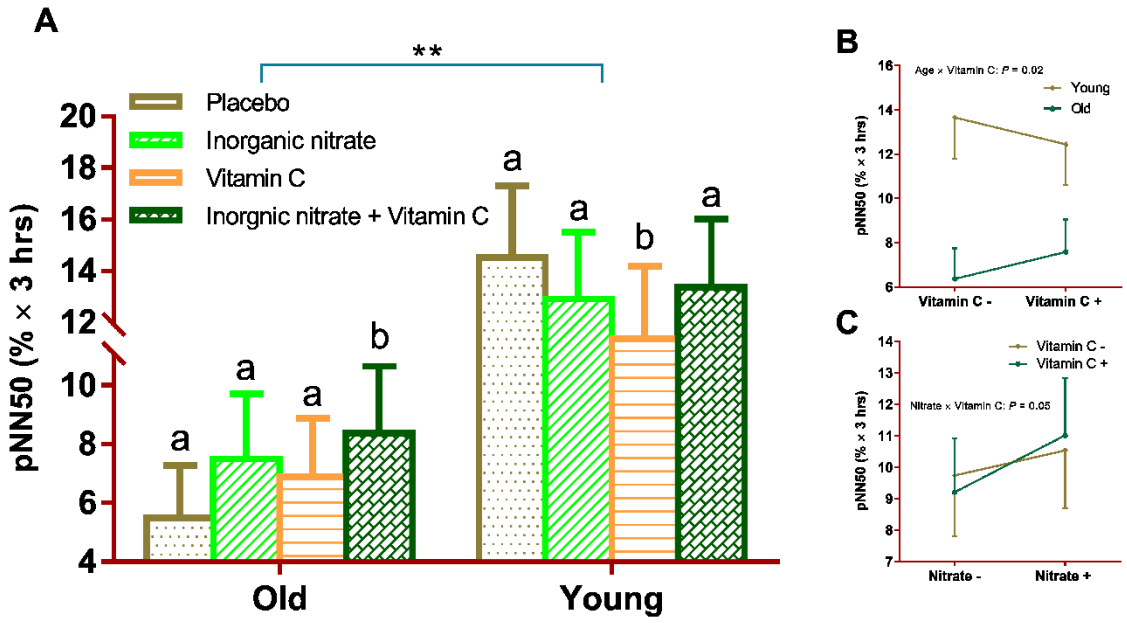


Figure 5.14: The percentage of intervals that differ from each other by more than 50ms (pNN50) over a full 3 hours recording (A), the age × vitamin C interaction (B) and inorganic NO_3^- × vitamin C interaction (C) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). **: P < 0.01

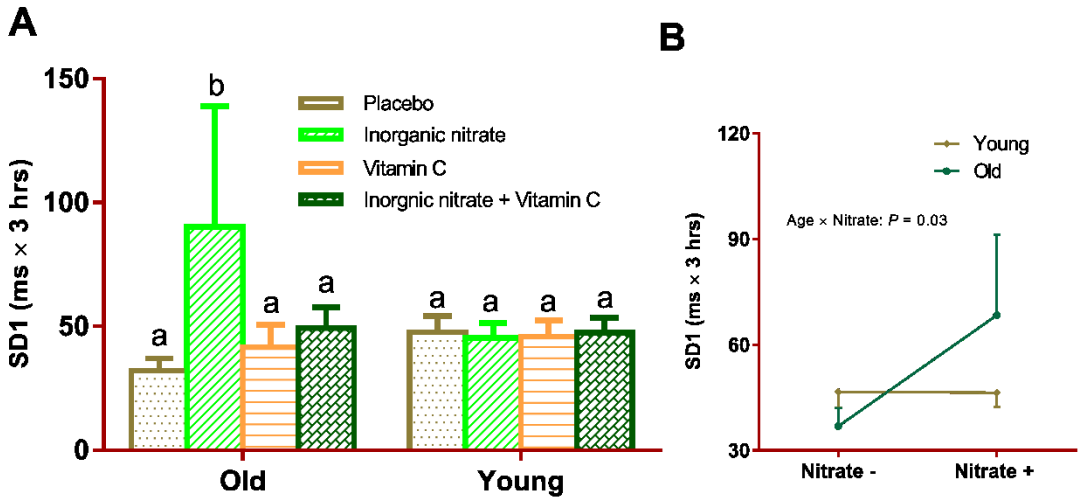


Figure 5.15: Standard deviation of the instantaneous RR variability (SD1) over a full 3 hours recording in younger (*n*= 10) and older participants (*n*= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). ms: millisecond.

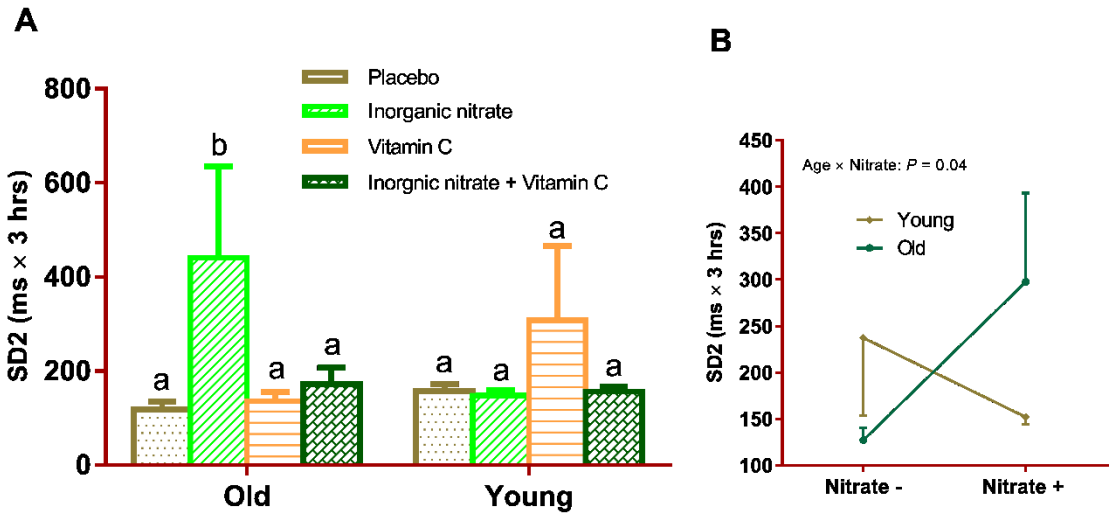


Figure 5.16: Standard deviation of the continuous variability of the heart rate (SD2) over a full 3 hours recording in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). ms: millisecond.

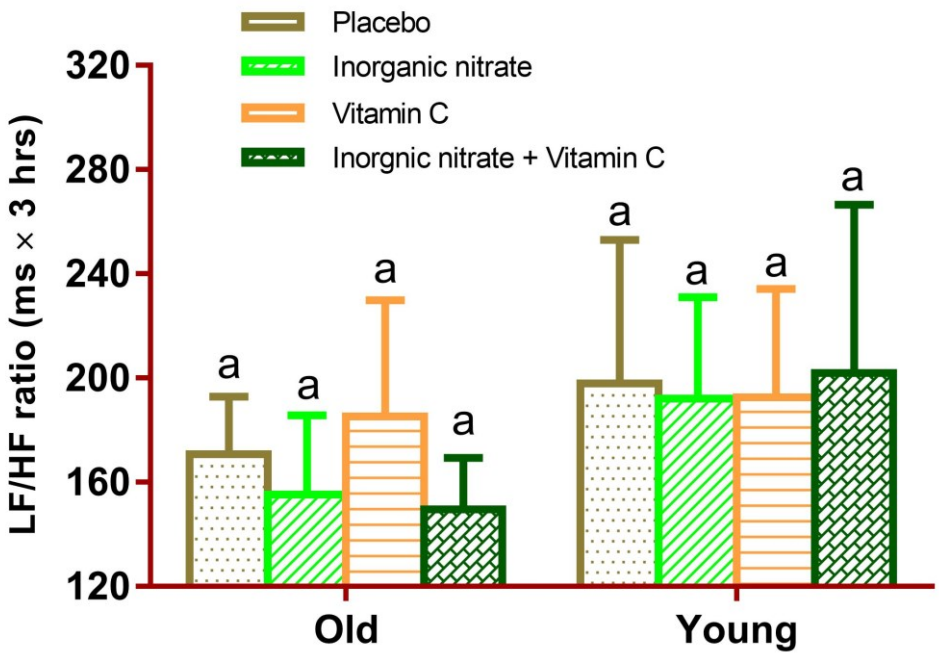


Figure 5.17: The low frequency/high frequency ratio (LF/HF) over a full 3 hours recording in younger (*n*= 10) and older participants (*n*= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). ms: millisecond.

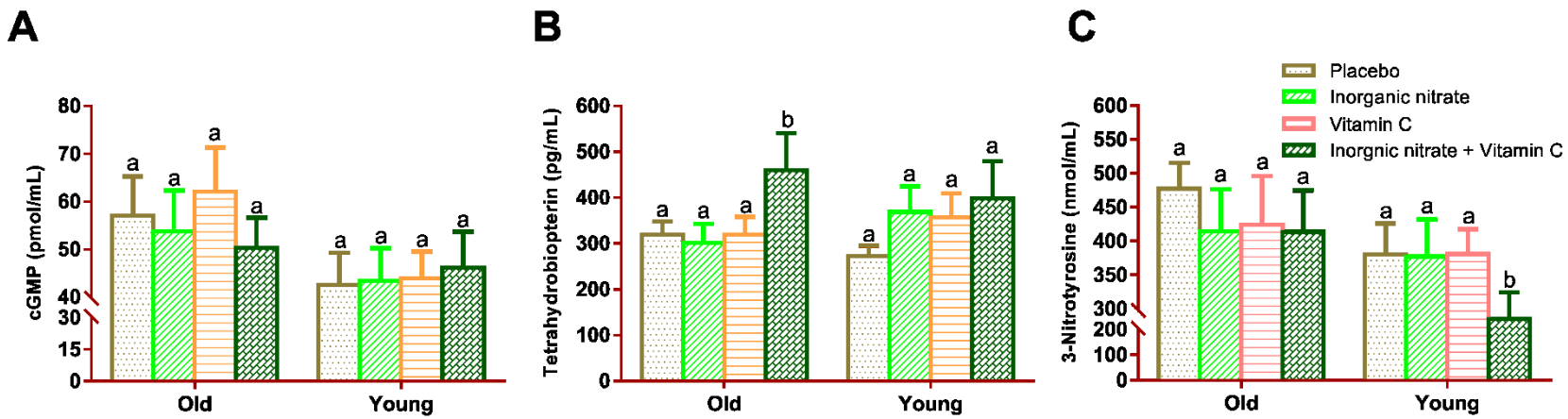


Figure 5.18: Plasma cyclic guanosine monophosphate (cGMP) (A) tetrahydrobiopterin (BH4) (B) and 3 nitrotyrosine (3-NT) (C) in younger (*n*= 10) and older participants (*n*= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05).

5.4 Discussion

5.4.1 *Summary of findings*

Higher BP and lower HRV indices observed in older participants. Nevertheless, the administration of inorganic NO_3^- and vitamin C, individually or combined, significantly improved the above outcomes in the same age group. Moreover, inorganic NO_3^- significantly modified the effects of vitamin C on diastolic BP and pNN50 index in younger participants. Finally, inorganic NO_3^- and vitamin C co-supplementation yielded synergistic effects on arterial stiffness indices (PWV and DC), HRV indices (RMSSD and pNN50) and circulatory biomarkers (BH4 and 3-NT).

5.4.2 *Effects on plasma NO_3^- and NO_2^-*

The administration of inorganic NO_3^- to our participants was associated with a significant increase in the concentrations of plasma NO_3^- and NO_2^-. In contrast to our previous study (Chapter 4), we did not observe a significant difference in plasma NO_3^- between younger and older participants. This suggests that adiposity and hyperglycaemia modified plasma NO_3^-/ NO_2^- concentrations in older people. It seems that, in older obese individuals, hyperglycaemia may have a detrimental effect on NO metabolites. In older participants, we observed a significant increase in 3-NT (biomarker of oxidative stress). This rise in oxidative stress may enhanced the conversion of NO_2^- and NO to $ONOO^-$ and then eventually to 3-NT (Bailey *et al.*, 2014; Teixeira *et al.*, 2016).

In the current study, we did not observe any modifying effects of vitamin C on plasma levels of NO_3^- and NO_2^-. This is in contrast with previous studies who showed a significant reduction in plasma S-nitrosothiols and NO_2^- after combined supplementation of flavonoids and inorganic NO_3^- (Bondonno *et al.*, 2012; Rodriguez-Mateos *et al.*, 2015).

5.4.3 *Effects on plasma vitamin C and DHA*

As expected, in both younger and older participants, plasma concentrations of vitamin C significantly increased after vitamin C administration. However, only in older participants, vitamin C intake caused significantly higher concentration of DHA. DHA is an oxidised product of ascorbic acid, it is formed at the end of protective antioxidant process of vitamin C (Sibmooh *et al.*, 2008). DHA can be regenerated back to ascorbic acid with the help of intracellular reducing agents such as glutathione or NADPH (Sibmooh *et al.*, 2008). Therefore, we may

speculate that age-related oxidative stress might enhance the oxidation of vitamin C to DHA. The other possible explanation is that the capacity of vitamin C to regenerate was compromised in older participants. Previous study in mice showed that ascorbic acid recycling capacity declined with age (Lykkesfeldt *et al.*, 1998).

5.4.4 ***Effects on BP***

We observed a significant reduction of -4 mmHg in systolic BP in older participants after single supplementation of inorganic NO_3^- or vitamin C. Furthermore, single or combined supplementation of inorganic NO_3^- and vitamin C significantly reduced mean BP (~-2.5 mmHg) in older individuals. Our results agreed with previous meta-analyses which showed significant reduction in systolic BP after vitamin C (-3.84 mmHg; 95% CI: -5.29, -2.38) and inorganic nitrate supplementation (-4.4 mmHg, 95% CI: -5.9, -2.8) (Juraschek *et al.*, 2012; Siervo *et al.*, 2013).

However, in the meta-analysis of vitamin C and blood pressure, Juraschek *et al.* (2012) observed a significantly higher reduction in systolic blood pressure in younger (age < 50 years) (-5.07 mmHg, 95% CI: -7.22 to -2.92) compared with older participants (age > 60 years) (-1.85 mmHg, 95% CI: -2.94 to -0.77). Nevertheless, nearly half of the studies included in that meta-analysis supplemented vitamin C with other micronutrients or pharmacological agents (Juraschek *et al.*, 2012). Therefore, we cannot rule out the modifying effects of age on the hypotensive effects of vitamin C.

In our study, the administration of vitamin C was associated with slight increase in BP in younger participants. However, in those participants, vitamin C supplementation was not associated with increase in oxidative stress markers (DHA and 3-NT). This suggests that the rise in BP may not have been a result of increased oxidative stress. Similarly, Magen *et al.* (2004) showed, in comparison with placebo, a significant increase of 4.2 mmHg in diastolic BP in hypertensive patients treated with vitamin C (500 mg/day) for 8 weeks. Another two studies also reported significant increase in diastolic BP (Title *et al.*, 2000; Ward *et al.*, 2005) with vitamin C administration. However, the latter two studies used a combination of vitamin C, vitamin E and folic acid (Title *et al.*, 2000) or polyphenols (Ward *et al.*, 2005). In contrast, in the present study, inorganic NO_3^- positively modified the effect of vitamin C on diastolic BP. Previous observational studies showed that 5-6 mmHg reduction in diastolic BP was associated with a 38 and 23% reduction in risk of stroke and CHD, respectively (Collins *et al.*, 1990).

5.4.5 *Effects on arterial stiffness*

Combined inorganic NO_3^- and vitamin C supplementation significantly improved arterial stiffness indices (PWV and DC) in older participants. The effects of inorganic NO_3^- + vitamin C was greater than the effects of both agents when given separately. This denote that the interaction of inorganic NO_3^- and vitamin C was synergistic. Previously conducted meta-analyses revealed no significant effects of inorganic NO_3^- or vitamin C supplementation on arterial stiffness indices (Chapter 2 and 3). Our finding of synergistic interaction of both dietary agent may have important health implications. However, due to the acute nature of the current study, this synergistic effect need to be confirmed in longer term clinical trials.

The mechanism of this synergism might be enhanced conversion of NO_2^- to NO. However, this synergism is not mirrored by an increase in cGMP concentration, the second messenger of NO. Moreover, we did not observe significant change in the concentration of NO_2^- after inorganic NO_3^- + vitamin C co-supplementation.

Previous inorganic NO_3^- studies showed conflicting results regarding cGMP concentration. For example, supplementing mice with inorganic NO_2^- was associated with increased (Bryan *et al.*, 2005) or no changes in cGMP concentration (Carlstrom *et al.*, 2010). In human study, Kapil *et al.* (2010a) showed that supplementing inorganic NO_3^- (24 mmol) to young male and female adults associated with significant increase in cGMP concentration. In a similar cohort, with a smaller dose of inorganic NO_3^- (8 mmol), Velmurugan *et al.* (2013) demonstrated a significant increase in cGMP in male volunteers only. Previous studies have shown that the vascular effects of NO are not completely dependent on cGMP production (Bolotina *et al.*, 1994; Jaffrey and Snyder, 2001).

Nevertheless, in our study, BH4 a co-factor of endogenous NO production increased significantly after the combined administration of both agents in a synergistic mode as well. BH4 depletion causes uncoupling of eNOS enzyme with the consequent production of superoxide free radicals instead of NO (Schmidt and Alp, 2007). Clinical studies have shown that BH4 supplementation may reverse endothelial dysfunction in healthy and in patients with cardio-metabolic disorders (Wang *et al.*, 2014). *In vitro* studies demonstrated that vitamin C may enhance the stability of BH4 and, therefore, maintain high BH4-to-BH2 ratio (Mortensen and Lykkesfeldt, 2014). The proposed mechanisms include the ability of vitamin C to reduce the oxidised BH3 to BH4 and/or by preventing BH4 from being oxidised, in the first place, by free radicals (Mortensen and Lykkesfeldt, 2014). Feeding wild-type mice diet containing 1% vitamin C significantly increased BH4 concentration in the aorta (d'Uscio *et al.*, 2003).

In addition to vitamin C, inorganic NO_3^- supplementation significantly increased BH4 concentration. Previous study in mice showed that the administration of inorganic NO_2^- significantly increased BH4 concentration and improved BH4-to-BH2 ratio (Stokes *et al.*, 2009). The proposed mechanism might be linked to the antioxidant effects of inorganic NO_3^-, therefore protecting BH4 from being oxidised or regenerating it from the oxidised form (BH2) (Stokes *et al.*, 2009). Therefore, besides being an external source of NO, our study showed the ability of inorganic NO_3^- to contribute to the endogenous NO production by increasing the concentration of BH4.

In our study, combined inorganic NO_3^- and vitamin C administration significantly reduced 3-NT concentration in younger participants. Like BH4, the combined supplementation showed synergistic interaction on 3-NT concentration. A study involved healthy volunteers showed that high dietary NO_3^- intake has no significant effect on plasma 3-NT concentrations (Pannala *et al.*, 2003). Likewise, inorganic NO_3^- did not significantly change the concentration of platelets NT of healthy adults (Richardson *et al.*, 2002). In our previous study (Chapter 4), inorganic NO_3^- administration significantly reduced the concentration of 3-NT. However, in that study, we included obese participants subjected to acute hyperglycaemia. Therefore, higher levels of oxidative stress were expected in their blood than the participants included in the current study. Similarly, vitamin C supplementation did not reduce 3-NT concentration significantly in healthy volunteers (Wray *et al.*, 2012). However, a significant reduction only seen in participants with higher oxidative stress (Ceriello *et al.*, 2013). In contrast, the present study showed that both inorganic NO_3^- and vitamin C when administrated together will significantly reduce 3-NT concentration in young healthy volunteers. This may signify potentiation of the effects of both agents when given together.

5.4.6 ***Effects on skin microvascular blood flow***

Marginal improvement in cutaneous microvascular blood flow was observed in younger and older participants after inorganic NO_3^- supplementation. Previous study demonstrated that supplementation of beetroot for three days to young healthy non-obese individuals significantly improved laser Doppler blood flow (Levitt *et al.*, 2015). However, 3 weeks supplementation of 6 mmol of inorganic NO_3^- in the form of beetroot to older (> 62 years) overweight and obese participants did not significantly improved cutaneous microvascular blood flow (Ashor *et al.*, 2015a). In line with above study, Gilchrist *et al.* (2013) did not find significant improvement in skin microvascular blood flow after 2 weeks of NO_3^--rich beetroot supplementation of diabetic patients. Nevertheless, our findings agree with the results of our meta-analysis testing the

effects of inorganic NO_3^- on EF which revealed a higher efficacy in healthy non-obese participants (Chapter 2).

5.4.7 *Effects on HRV indices*

Inorganic NO_3^- significantly improved HRV indices (SD1 and SD2) in older but not younger participants. Low HRV variability is regarded as an important predictor of mortality and morbidity in patients with acute myocardial infarction (Huikuri and Stein, 2012). Moreover, HRV indices considered to be risk factors for all-cause, cardiac and arrhythmic deaths (Huikuri and Stein, 2012). Previous study demonstrated that inorganic NO_3^- significantly increase SDNN in healthy African American female adults (Bond *et al.*, 2014). However, recent studies did not report a significant improvement of HRV indices after the administration of inorganic NO_3^- (Alsop and Hauton, 2016; Ashor *et al.*, 2016a). The proposed mechanisms of increased HRV with inorganic NO_3^- might be related to the ability of NO to enhance parasympathetic and reduced sympathetic activities of the heart (Sartori *et al.*, 2005).

Combined inorganic NO_3^- and vitamin C supplementation demonstrated a synergistic interaction in raising RMSSD and pNN50 in older participants. The antioxidant and NO-sparing properties of inorganic NO_3^- and vitamin C might be the reason of this improvement in HRV (Piccirillo *et al.*, 2003; Monahan *et al.*, 2004; Bond *et al.*, 2014). Previous studies suggest that oxidative stress and reduction in NO availability contribute mechanistically to the age-associated reduction in HRV (Monahan *et al.*, 2004). Moreover, a study in rabbits claimed that vitamin C administration in the absence of oxidative stress has no influence on HRV indices (Z. Li *et al.*, 1996). Therefore, it seems that inorganic NO_3^- and vitamin C significantly improved HRV in older participants by reducing oxidative stress and increasing NO availability. Previous study showed that the acute infusion of vitamin C to eight young (23 years) and seven older (63 years) individuals was associated with a significant improvement in HRV in older but not younger participants (Monahan *et al.*, 2004).

5.4.8 *Public health implications*

In older participants, systolic BP was reduced by -4 mmHg following inorganic NO_3^- or vitamin C supplementation. It has been suggested that, in moderately hypertensive individuals, a 5 mmHg reduction in BP might reduce the incidence of stroke by 22% and CHD by 16% and prevent up to 75,000 deaths per year (Vanhatalo *et al.*, 2010). Moreover, we should take into consideration that our participants were healthy, well-nourished and with normal BP.

Therefore, a reduction of -4 mmHg in systolic BP might have greater benefits in population at risk e.g. obese, undernourished or individuals with underlying cardiovascular diseases. Inorganic NO_3^- and vitamin C are common in our everyday diet and both have been considered to possess beneficial cardiovascular effects. Therefore, studying the possibility of interaction of these two dietary agents on cardiovascular markers allow for a better understanding of their beneficial roles in our normal diet. In our study, inorganic NO_3^- + vitamin C displayed a reduction of -2 m/s in PWV in older participants. Previous meta-analysis of 17 longitudinal studies (15,877 subjects, followed-up for a mean of 7.7 years) showed that every 1 m/s increase in aortic PWV is associated with 14% increase in the risk of cardiovascular events and 15% increase in the risk of CVD mortality (Vlachopoulos *et al.*, 2010).

5.4.9 ***Strengths and limitations***

The dietary factors inorganic NO_3^- and vitamin C are abundant in our diet and are frequently co-ingested in our meals. In the current study, we have demonstrated for the first time, the potential of beneficial synergistic interaction of inorganic NO_3^- and vitamin C on markers of CVDs. Moreover, these beneficial effects are further augmented in older population. The crossover Latin-square design ensures a strict within-volunteer control of factors that may affect the outcomes such as age, body composition and health status. In the current study, we included a selected population of healthy normal/overweight population with no diseases or drug treatments that may confound our results. Moreover, the presence of two separate age groups helped in understanding the modifying effects of age on our interventions. However, there are some limitations associated with the study need to be addressed in future clinical trials. We did not investigate the possibility of interactions of vitamin C with inorganic NO_3^- in the oral cavity (no saliva samples collected), stomach (expelled stomach NO was not collected) and urinary system (no urine samples collected). Moreover, we did not use FMD and carotid-femoral PWV; these regarded as the gold standard measures of endothelial function (Inaba *et al.*, 2010) and arterial stiffness (Ben-Shlomo *et al.*, 2014), respectively.

5.4.10 ***Conclusions***

Age was a modifier of the effects of vitamin C and inorganic NO_3^- on markers of CVD risk. Furthermore, inorganic NO_3^- and vitamin C co-supplementation yielded greater effects than each intervention alone on arterial stiffness and HRV indices and circulatory biomarkers. Inorganic NO_3^- qualitatively modified the effects of vitamin C on diastolic BP and HRV. Since these micronutrients complement each other by working on different molecular pathway,

combined administration of inorganic NO_3^- and vitamin C may have additive or synergistic interaction on vascular health. Moreover, to avoid the adverse effects linked to higher doses of inorganic NO_3^- and vitamin C, co-supplementation of these micronutrients in lower doses might produce greater beneficial effects than each intervention alone. Future studies should focus on addressing the mechanisms and the potential sites of interactions of vitamin C and inorganic NO_3^-.

Chapter 6. General discussion and conclusions

6.1 Overview

Cardiovascular mortality varies between countries; relatively low mortality rates are observed in Japan and Mediterranean countries in comparison with other high-income countries (Lidder and Webb, 2013). Nutritional and life-style factors (i.e., anti-oxidants, essential fatty acids, salt intake, physical inactivity, stress) significantly affect the incidence and prevalence of CVD (Kapil *et al.*, 2010b). Epidemiological studies have consistently showed that a balanced diet rich in fruits and vegetables lowers CVD risk (Crowe *et al.*, 2011). Such foods contain multiple bioactive components which can influence a wide range of CVD risk factors including blood lipids, insulin sensitivity, oxidative stress, inflammation and ED (Shen *et al.*, 2015). Reduced NO availability is one of the primary hallmarks of ED (Hobbs *et al.*, 2013). In addition, the discovery of the role of NO in the regulation of EF, angiogenesis and platelet aggregation contributed to the deeper understating of the physiological and biomolecular mechanisms preceding the development of atherosclerosis and CVD (Nichols *et al.*, 2013). Inorganic NO_3^- may represent a key dietary component linked to the health benefits of Japanese and Mediterranean diets (Lidder and Webb, 2013) and explained by an increased availability of NO derived from the non-enzymatic NO_3^- - NO_2^- -NO pathway (Lundberg *et al.*, 2006).

Vitamin C is an essential nutrient which has antioxidant roles in the human body (Padayatty *et al.*, 2003). It is regarded by some investigators as the most important antioxidant in human plasma (Halliwell, 1996). Ascorbic acid (the reduced form of vitamin C) scavenges physiologically relevant reactive oxygen and nitrogen species (Carr and Frei, 1999). Evidence from observational studies demonstrating associations between higher vitamin C intake (or status) and better cardiovascular health (Fletcher *et al.*, 2003). Meta-analysis of data from 9 cohort studies showed that vitamin C supplementation at doses exceeding 700 mg/day was associated with 25% reduction in coronary heart disease risk (Knekt *et al.*, 2004).

Advanced age is regarded as a major risk factor for CVD (Sindler *et al.*, 2011). Ageing is associated with complex structural and functional changes in the vascular system including ED and increased arterial stiffness (Jani and Rajkumar, 2006). BH4 and L-arginine, two key cofactors in NO production, are reduced in skeletal muscle arterioles with increasing age (Delp *et al.*, 2008). Moreover, the low concentration of NO_2^- found in the arteries, heart, and plasma of aged mice was successfully reversed by the administration of inorganic NO_2^- (Sindler *et al.*, 2011). Therefore, development of nutritional strategies to increase NO availability in

endothelial cells and so attenuate vascular ageing could positively impact on CVD risk and improve the quality of life and life expectancy of older people (El Assar *et al.*, 2012).

6.2 Inorganic NO_3^- and EF

In the second chapter of this thesis, I examined published evidence regarding the efficacy of inorganic NO_3^- and beetroot supplementation on EF. Additionally, I investigated whether the effect of NO_3^- on EF was modified by the participants' characteristics (age, health status, baseline BMI and baseline BP) or study characteristics (design, dose and duration of inorganic NO_3^- supplementation). I found that supplementation with inorganic NO_3^- or beetroot resulted in significant improvement in FMD and that the improvement in EF tended to be greater in younger, non-obese and healthy participants. However, neither PWV nor AIx was improved by these interventions. Nevertheless, inorganic NO_3^- or beetroot administration in larger doses might be associated with greater reduction in PWV (Figure 2.5, Chapter 2).

6.3 Vitamin C and EF

Vitamin C is a powerful antioxidant that may mitigate the earliest stages of atherosclerosis through several mechanisms (Aguirre and May, 2008). One of these mechanisms is the ability of vitamin C to augment NO availability (May and Harrison, 2013). Vitamin C inactivates superoxide free radicals, stabilizes BH4, enhances eNOS activity and preserves L-arginine and cGMP (May, 2000). All the above factors are important for endogenous production of NO (Forstermann, 2010). Moreover, vitamin C may enhance NO production through the exogenous (dietary) NO_3^- - NO_2^- - NO pathway (Hord *et al.*, 2009).

In the third chapter of my thesis, I conducted an umbrella review which investigated the effects of vitamin C supplementation on markers of cardiovascular diseases (i.e. arterial stiffness, blood pressure, EF, glycaemic index and lipid profile). My secondary aim was to explore the factors that may modify the effects of vitamin C on these cardiovascular markers. The included studies revealed little evidence for an overall effect of vitamin C supplementation on markers of CVD risk. However, subgroup and meta-regression analyses indicated significant benefits in older participants and in those with higher BMI, lower vitamin C status and at higher CVD risk.

6.4 Impact of inorganic NO_3^- on adverse cardio-metabolic effects induced by acute hyperglycaemia in younger and older obese participants

Ageing, obesity and hyperglycaemia are major risk factors for CVD (Ahima, 2009). The adverse effects of these risk factors may be mediated by oxidative and inflammatory stress which causes endothelial dysfunction by decreasing NO availability (El Assar *et al.*, 2012; Mah and Bruno, 2012). Reactive oxygen species deplete arginine, oxidise BH4 and enhance ADMA, consequently causing eNOS uncoupling which produces free radicals and further reduces NO availability (Mah and Bruno, 2012).

In chapter four of my thesis, I conducted a crossover RCT aimed at testing whether the administration of inorganic NO_3^- could ameliorate the acute adverse effects of postprandial hyperglycaemia on vascular function in obese participants. I also tested the hypothesis that the response to the NO_3^- administration was age-dependent with potentially greater benefits in older than in younger participants. Following inorganic NO_3^- supplementation, I observed a two-fold higher plasma NOx concentrations in young compared with older participants. Moreover, significantly lower concentrations of cGMP and thrombomodulin and higher concentration of ICAM-3 were observed in older compared to younger participants. This may reflect a state of greater inflammation and oxidative stress in the older participants. Supplementation with inorganic NO_3^- produced greater improvements in biomarkers of inflammation (IL-6) and oxidative stress (3-NT) in older compared with young participants. Conversely, greater improvement in biomarkers of endothelial function (P- and E-selectin) was observed in younger rather than older participants. However, no changes in cutaneous microvascular blood flow was observed in either age group. Therefore, the findings from this study imply that younger and older participants may respond differently to a single dose of inorganic NO_3^- in the presence of acute hyperglycaemia. In older participants, oxidative stress (increased 3-NT) and inflammation (increased IL-6) elicited by acute hyperglycaemia was ameliorated by the co-administration of a single dose of inorganic NO_3^-. In contrast with 3-NT and IL-6, in older participants, non-significant changes in E selectin and P selectin concentrations were observed after the glucose challenge. These blunted responses might reflect the ED in the arteries of older participants and might explain the absence of effects of inorganic NO_3^- on these biomarkers.

6.5 Effects of inorganic NO_3^- and vitamin C in younger and older participants

Evidence presented in Chapters 2 and 3 showed that supplementation with either inorganic NO_3^- or vitamin C may improve markers of EF in certain circumstances. Both dietary factors have the potential to improve NO availability (May, 2000; Hobbs *et al.*, 2013) and both have anti-inflammatory and antioxidant functions (Aguirre and May, 2008; Ashor *et al.*, 2016a). However, the effects of supplementation with each food component depended on the characteristics of the included population. While the effects of vitamin C were greater (or detectable only) in older, obese participants and in those with underlying diseases (Ashor *et al.*, 2014a; Ashor *et al.*, 2015b), the effects of inorganic NO_3^- were larger in younger, non-obese and healthy participants (Lara *et al.*, 2015).

In Chapter 5, I reported the findings from a 2x2 factorial crossover RCT in healthy non-obese young and older adults which was designed to investigate the potential additive or synergistic effects of vitamin C and inorganic NO_3^- on BP, HRV and vascular function. The secondary aim was to determine whether age modified the effects of vitamin C and inorganic NO_3^- interventions. In contrast with results from studies in obese participants (reported in Chapter 4), there was no difference in plasma NO_3^- response to oral supplementation in the non-obese participants. I observed significant increase in plasma NO_2^- after inorganic NO_3^- supplementation, and this effect was not modified by vitamin C co-administration. However, inorganic NO_3^- + vitamin C showed synergistic effects on arterial stiffness, HRV indices and circulatory biomarkers. Still, we did not observe similar synergistic effects in BP, skin microvascular blood flow and cGMP concentration. Moreover, vitamin C showed different effects on BP in young and older participants. Supplementation with this vitamin tended to reduce BP in older participants but increase it in younger participants. Similar findings were also observed in HRV index (pNN50). However, these unexpected effects of vitamin C supplementation on BP in younger participants appeared to be mitigated by the co-administration of inorganic NO_3^-.

6.6 Personalized approach to nutritional interventions

Personalized medicine or precision medicine is the treatments that targeted to the needs of individual patients based on genetic, biomarker, phenotypic, or psychosocial characteristics that distinguished a given patient from other patients with similar clinical presentation (Jameson and Longo, 2015). The aim of personalized medicine is to improve the clinical outcomes for individual patients and minimizing unnecessary side effects for those less likely to have a

response to a specific treatment (Jameson and Longo, 2015). In the first two chapters, we observed that participants' characteristics such as age, BMI or health status significantly modified the effects of inorganic NO_3^- or vitamin C on cardiovascular diseases markers. Meta-analysis of RCTs (Chapter 2) revealed that inorganic NO_3^- significantly improved EF. However, these effects tend to be greater in younger, healthier, non-obese populations. Several factors modulate the effects of vitamin C on CVD markers (Chapter 3). Meta-analysis studies reported greater improvement of EF in older, obese population (Chapter 3). Moreover, dose, duration and health status significantly modified the effects of vitamin C on CVD markers (Chapter 3).

On the other side, in the RCTs reported in chapter 4 and 5 we used age as phenotypic characteristic that dictate the personalized approach with our interventions. We noticed that age significantly modified the effects of inorganic NO_3^- and vitamin C on cardiovascular outcomes. In the inorganic NO_3^- and hyperglycaemia RCT (Chapter 4), significant improvements in biomarkers of EF were seen in younger participants only. Moreover, for the first time, this RCT showed that inorganic NO_3^- could ameliorate oxidative- and inflammatory stress induced by hyperglycaemia in older obese participants. Likewise, in the RCT of inorganic NO_3^- and vitamin C (Chapter 5), I observed that age significantly modified the effects of vitamin C on BP and HRV indices.

Results from my thesis emphasized the importance of a personalized approach for increasing the efficacy of dietary interventions with vitamin C and inorganic NO_3^-. Furthermore, my results may partially explain the failure of antioxidant vitamins to show cardiovascular protective effects in large scale interventional studies. Recruiting individuals with diverse phenotypic and metabolic profiles may dilute any potential protective effects in subgroups of population who may benefit more from these interventions. Therefore, future studies should implement a careful selective recruitment strategy, informed by evidence-based literature synthesis to maximize the potential benefits of nutritional interventions.

6.7 Inorganic NO_3^- and vitamin C: looking beyond NO availability

Inorganic NO_3^- and vitamin C supplementation significantly reduced BP and increased HRV indices. However, these changes in BP and HRV were not mirrored with an increase in cGMP concentration suggesting that some of the beneficial effects of these dietary agents were cGMP-independent. Inorganic NO_3^- significantly reduced inflammatory and oxidative stress markers in younger and older obese individuals in response to hyperglycaemia (Chapter 4). Moreover,

inorganic NO_3^- marginally reduced oxidative stress marker concentrations in younger and older non-obese participants (Chapter 5). Therefore, our two RCTs demonstrated that inorganic NO_3^-, besides being an exogenous source of NO, may have anti-inflammatory and free radical scavenging properties.

Exploring the mechanisms of action of inorganic NO_3^- and vitamin C is beyond the scope of the present study. However, the following are speculations about the proposed mechanism of the beneficial effects of both interventions on BP and vascular function. Bryan *et al.* (2005) demonstrated that some of the effects of circulatory NO_2^- are NO-independent. The authors showed the ability of NO_2^- to modulate signaling pathways by enhancing gene expression or by post-translational modification of proteins (Bryan *et al.*, 2005). Additionally, previous studies have shown that the vascular effects of NO are not completely dependent on cGMP production (Bolotina *et al.*, 1994; Jaffrey and Snyder, 2001).

Previous study showed that pharmacological doses of vitamin C increased hydrogen peroxide (H_2O_2) production (Chen *et al.*, 2007). Furthermore, experimental studies demonstrated that H_2O_2 has vasodilator properties independent of NO pathway (Feletou *et al.*, 2010). The mechanisms of the H_2O_2-induced vascular relaxation involve stimulation of endothelium derived hyperpolarizing factors (EDHFs) (Feletou *et al.*, 2010). In an *in vitro* study of rabbit iliac arteries, Garry *et al.* (2009) showed that vitamin C and BH4 induced relaxation of vessel wall by NO-independent, EDHF-dependent mechanisms. Moreover, the relaxation was abolished by the addition of H_2O_2 inhibitor (catalase) (Garry *et al.*, 2009).

Evidence of negative responses to vitamin C in the younger participants appears to support the emerging notion of a potential beneficial role of free radicals in vascular protection. In addition to direct vasodilatory properties, free radicals may also play an important role in the upregulation of endogenous antioxidant capacity through increased expression of several enzymes known to "detoxify" free radicals (Liu *et al.*, 2003; Ristow *et al.*, 2009). Therefore, we speculate that, in younger participants, where pro-oxidant and antioxidant forces are somewhat balanced, the aggressive reduction in free radical concentration after vitamin C administration may have removed or suppressed certain oxidative species that possess some beneficial properties in terms of vascular regulation, resulting in the observed increase in BP.

6.8 Public health implications

In contrast to the unsatisfactory findings from large-scale clinical trials concerning the beneficial effect of chronic antioxidant vitamins administration on cardiovascular health

(Honarbakhsh and Schachter, 2009), the present study has identified an acute improvement in markers of cardiovascular diseases. However, the difference in design between acute and long-term interventional studies limits a direct comparison with data from these previous studies. Nevertheless, using this short-term, interventional design has allowed us to demonstrate the ability of our interventions to reverse the age-associated deterioration in physiological markers in older participants without the additional confounding variables associated with longer-term treatment studies (Michels and Frei, 2013).

Findings from my PhD thesis provide important mechanistic and physiological insights regarding the link between endothelial dysfunction and oxidative stress in the elderly and demonstrate the ability of these single-dose acute interventions to improve cardiovascular biomarkers. Moreover, inorganic NO_3^- and vitamin C are abundant in our diet and usually consumed together in everyday meals. Therefore, studying the interaction between these nutrients is vital to our understanding of the mechanisms of their beneficial effects in diet. After all, due to the acute nature of the current studies, we cannot support at this stage the beneficial effects of inorganic NO_3^- and vitamin C on cardiovascular health, which awaits confirmation in longer-term clinical trials.

Previously research concentrated on studying the effects of individual components of diet on cardiovascular health (e.g. antioxidant vitamins, polyphenols or inorganic NO_3^-) (Hooper *et al.*, 2008; Myung *et al.*, 2013; Siervo *et al.*, 2013). While it is crucial to study the effects of individual components, it is uncertain whether consumption of a single active dietary component will have the same beneficial effects as when it is consumed as part of a whole food or combination of food (Bondonno *et al.*, 2015a). Combined administration of micronutrients may have additive or synergistic interaction due to the fact that these micronutrients complement each other by working on different molecular pathway (Bondonno *et al.*, 2015a).

We detected a -4 mmHg reduction in systolic BP with inorganic NO_3^- or vitamin C supplementations. In moderately hypertensive individuals, a 5 mmHg reduction in BP might reduce the incidence of stroke by 22% and CHD by 16% and prevent up to 75,000 deaths per year (Vanhatalo *et al.*, 2010). Moreover, we should take into consideration that our participants were healthy, well-nourished and with normal BP. Therefore, a reduction of -4 mmHg in systolic BP might be more augmented in population at risk e.g. obese, undernourished or individuals with underlying cardiovascular diseases. Moreover, combined supplementation with inorganic NO_3^- and vitamin C determined a reduction of -2 m/s in PWV (Chapter 5). Previous meta-analysis of 17 longitudinal studies (15,877 subjects, followed-up for a mean of

7.7 years) showed that every 1 m/s increase in aortic PWV is associated with 14% increase in the risk of cardiovascular events and 15% increase in the risk of CVD mortality (Vlachopoulos *et al.*, 2010). Another meta-analysis of 18 longitudinal cohort studies (8169 participants; mean follow-up, 3.6 years) showed that every 1 m/s increase in brachial-ankle PWV is associated with 12% increase in the risk of cardiovascular events and 13% increase in the risk of CVD mortality (Vlachopoulos *et al.*, 2012). We observed, in older participants, a marked increase in HRV indices (SD1 and SD2) after inorganic NO_3^- supplementation (Chapter 5). In a cohort of 5,272 participants aged 55 years and followed up for 4 years, investigators found that subjects in the lowest quartile of SDNN had 80% increase in the risk of cardiac mortality (hazard ratio = 1.8, 95% CI: 1 to 3.2) (de Bruyne *et al.*, 1999). Another cohort study involved 605 individuals aged between 50 and 75 years followed-up for 9 years revealed that impaired SDNN was associated with an approximately 70% increase in the risk of all-cause mortality in subpopulation with diabetes (Gerritsen *et al.*, 2001). Therefore, the ability of inorganic NO_3^- to improve HRV indices may have a significant impact on cardiovascular health.

6.9 Strengths and limitations of experimental strategy

In Chapter 4, we investigated for the first time, the effects of a single dose of inorganic NO_3^- on hyperglycaemia-induced ED in both younger and older participants. The study had a robust experimental design (randomised, double-blind and cross-over) and to avoid a carry-over effect, we allowed one week wash-out period between each intervention. Previous study demonstrated that the concentrations of NO metabolites return to baseline levels after 24 h of consumption (James *et al.*, 2015). In Chapter 5, the crossover Latin-square design ensures a strict within-volunteer control of factors that may affect the outcomes such as age, body composition and health status. In that study, we included a selected population of healthy normal/overweight population with no diseases or drug treatments that may confound our results. Moreover, the presence of two separate age groups helped in understanding the modifying effects of age on our interventions.

However, these crossover RCTs reported in Chapters 4 and 5 have several limitations. Both studies were acute studies with small sample size. We do not know whether the effects of intervention observed in these studies will last longer in chronic intervention trials. It was difficult to compare the concentrations of NO_2^-/NO_3^- between the two studies due to the diverse methods used in handling the blood samples and the diverse method used in laboratory analysis. In the work reported in Chapter 4, due to logistic issues, blood samples processing were not immediately carried out after collection. Additionally, due to financial issues, we used

the available GC-MS method which was not sensitive enough to detect low concentration of NO_2^-. However, in the inorganic NO_3^- and vitamin C study we processed the blood directly after collection and we used Sievers NO analyser which is regarded as the gold-standard method for measuring NO_2^-/NO_3^- in plasma (Pelletier *et al.*, 2006).

There are limitations associated with using LDF assessment of EF in our clinical trials. First, is the inability to determine the absolute blood flow value with LDF (Turner *et al.*, 2008). LDF implements a semi-quantitative assessment of skin blood flow and is thus expressed in arbitrary PU (Turner *et al.*, 2008). Second, the contribution of other molecules besides NO to the vasodilation associated with LDF. These molecules include prostaglandin I_2 and EDHF (Kvandal *et al.*, 2003; Holowatz *et al.*, 2005). However, a recently conducted study excludes the possibility of contribution of prostaglandins to the vasodilatory response of PORH (Hellmann *et al.*, 2015). Lastly, we did not measure NO-independent vasodilation using sodium nitroprusside due to a single chamber use. Therefore, we cannot exclude the possibility of structural changes in the vessel wall that may cause reduction in the vasodilator capacity of the vessels (Turner *et al.*, 2008).

6.10 Future work

6.10.1 *Long-term effects of inorganic NO_3^- and vitamin C*

In Chapter 2 and 3 of this thesis, most of the studies included in the systematic reviews and meta-analyses had a short-duration. For example, the study with the longest duration investigating the effects of inorganic NO_3^- on BP and EF was 6 weeks (Velmurugan *et al.*, 2016). Similarly, the longest studies investigating the effects of vitamin C on BP (Fotherby *et al.*, 2000) and EF (Schindler *et al.*, 2003; Magen *et al.*, 2004) were 12 and 8 weeks, respectively. However, these studies, together with our two crossover RCTs, cannot verify whether inorganic NO_3^- and vitamin C can sustain their efficacy in the long term. Therefore, the next step is to investigate the effects of these dietary agents on cardiovascular outcomes in longer-duration RCTs.

6.10.2 *Effects of inorganic NO_3^- and vitamin C in clinical population*

In our crossover RCTs, our targets were obese and non-obese, healthy younger (18-40 years) and older (55-70 years) population. Therefore, it is unknown whether our interventions, inorganic NO_3^- and vitamin C, will have similar effects in the elderly (> 70 years) or in populations with underlying cardiovascular diseases. Targeting those populations may have

much larger public health implications and may contribute to further improvements in quality of life.

6.10.3 *Effects of hyperglycaemia on NO metabolites*

We observed that plasma NOx concentration was double at baseline and after inorganic NO_3^- supplementation in younger compared with older obese participants subjected to acute hyperglycaemia (Chapter 4). In contrast, in the work reported in Chapter 5 which involved non-obese younger and older participants (with no glucose challenge); there was no significant difference in plasma NO_3^- concentrations at baseline and after inorganic NO_3^- supplementation. This suggests that adiposity and hyperglycaemia modified plasma NO_3^-/ NO_2^- concentrations in older people. It seems that, in older obese individuals, hyperglycaemia may have a detrimental effect on NO metabolites. The heightened oxidative stress in older participants observed post-glucose challenge (Chapter 4) might be the culprit of the reduction in plasma NO_3^- concentration. I suggest conducting a study of older obese and non-obese individuals. These participants will be subjected to acute hyperglycaemia and supplemented with a single dose of inorganic NO_3^-. The aim will be to study the effects of acute hyperglycaemia on NO metabolites and oxidative stress markers with and without NO_3^- supplementation. The secondary aim will be to investigate whether obesity modify the effects of inorganic NO_3^- supplementation on the study outcomes.

6.10.4 *Dose-response relationship of inorganic NO_3^- and vitamin C*

In my vitamin C and inorganic NO_3^- study (Chapter 5), I used fixed doses of inorganic NO_3^- (7 mg/kg) and vitamin C (20 mg/kg). Previous study of flavanols and inorganic NO_3^- supplementation reported a synergistic interaction at lower doses of both dietary factors rather than higher doses (Rodriguez-Mateos *et al.*, 2015). Therefore, we recommend an intake-response study of inorganic NO_3^- and vitamin C. In this study, we will use different doses of both inorganic NO_3^- and vitamin C to find out the optimal combinational doses that give the most beneficial effects, if any, on BP and EF. Furthermore, in addition to plasma, the concentrations of NO metabolites should be investigated in urine, saliva and stomach to verify the potential site of interaction of both interventions.

6.11 Conclusions

Data synthesis of RCTs revealed significant improvement of measures of EF after supplementation with (often large doses of) inorganic NO_3^- and vitamin C for (usually) short time periods. However, participants' characteristics such as age, BMI and health status significantly modified the effects of the above interventions. Inorganic NO_3^- tended to improve EF to a greater extent in younger, non-obese and healthy participants. In contrast, vitamin C seems to show greater improvement in older, obese and participants with higher CVD risk. These results emphasise the importance of a personalised approach to interventions with inorganic NO_3^- and vitamin C when attempting to enhance primary or secondary prevention of cardiovascular diseases. However, most of the detailed information of effects of inorganic NO_3^- or vitamin C supplementation on markers of EF have been derived from studies with small sample sizes and of short duration. Researchers should take these limitations in consideration when designing future studies.

As anticipated, an acute glucose challenge increased inflammatory, oxidative stress markers and deteriorated NO availability and EF in both younger and older obese individuals. This provided a responsive experimental paradigm in which to demonstrate the acute effects of a single dose of inorganic NO_3^- in counteracting the adverse effects of hyperglycaemia. Greater improvement in biomarkers of EF was observed in younger participants. On other hand, older participants showed greater reduction in oxidative stress and inflammatory markers. These findings offer novel research questions to be investigated in future studies. Moreover, if confirmed in longer interventions, these findings could have important implications for the prevention and management of pathological processes associated with post-prandial hyperglycaemia.

There is a potential of additive or synergistic interaction of inorganic NO_3^- and vitamin C on markers of CVDs that may have public health implications. The combination of inorganic NO_3^- and vitamin C may have greater beneficial effects on cardiovascular outcomes than the supplementation of individual agents. The aim of future research is to investigate whether low doses of inorganic NO_3^- and vitamin C will achieve greater cardiovascular protection than larger doses of these dietary agents given individually. Using low doses of inorganic NO_3^- and vitamin C in combination will alleviate the adverse effects associated with larger doses of these dietary agents.

Appendix A. Ethical approval of the inorganic NO_3^- and hyperglycaemia study

NHS
Health Research Authority
National Research Ethics Service

NRES Committee Yorkshire & The Humber - Humber Bridge
HRA NRES Centre North West
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7816
Facsimile: 0161 625 7299

13 August 2013

Mr Shakir Chowdhury
Campus for Ageing and Vitality
Newcastle University
Newcastle on Tyne
NE4 5PL

Dear Mr Chowdhury

Study title:	**Effects of inorganic nitrate administration on glycaemic control and oxidative stress in young and older-aged obese subjects: a cross-over, double-blind randomised clinical trial**
REC reference:	**13/YH/0253**
IRAS project ID:	**129696**

The Research Ethics Committee reviewed the above application at the meeting held on 31 July 2013. Thank you for attending to discuss the application.

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 Co-ordinator Miss Diane Catterall, nrescommittee.yorkandhumber-humberbridge@nhs.net.

Ethical opinion

The Committee welcomed you and Mr Chowdhury to the meeting and asked you to outline the ethical issues.

> *The researchers advised the Committee that the cannula insertion is slightly invasive. They confirmed that this is not the first time this has been studied; another study has been conducted that involved inorganic nitrate in young healthy subjects that did not find any affect, although this has not been published. The dosage of inorganic nitrate would be compared to that of a big bowel of rocket.*
>
> The Committee questioned the rationale behind recruiting patients with Type 2 Diabetes on a diet, and asked whether this could skew the results.
>
> *The researchers explained that there are different peaks in glucose, and they will be comparing them to each other. Statistically they do not think it will affect the results. They will find out during the course of the research and report it at the end.*
>
> The Committee questioned the possibility of a conflict of interest, given that the student works for Herbalife as an Independent Sales Distributor/Advisor.

The Chief Investigator told the Committee that there is no relationship with the student and the project, the project was already funded and developed, and he wants the project to be scientifically independent with no outside influence.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Other conditions specified by the REC

1. The Participant Information Sheet should be amended as follows:
    a. Amend the word 'Obese' to 'Overweight'
1. The Committee would like to see the Consent Form revised as follows:
    a. Amend point 4 to read: 'I give permission for my samples to be stored for up to 10 years and then destroyed, and with the appropriate research ethics approval, retained samples may be stored and used in future research studies'
    b. Include the following mandatory statement 'I understand that relevant data collected during the study, may be looked at by individuals from [company name], from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to this data.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Advertisement	Flyer 1	04 July 2013
Advertisement	Leaflet 1	04 July 2013
Covering Letter	from Dr Mario Siervo	16 July 2013
Evidence of insurance or indemnity		06 July 2013
Investigator CV	Dr Mario Siervo	04 July 2013
Investigator CV	Dr Jose Lara	04 July 2013
Investigator CV	Dr Gabriele Saretzki	04 July 2013
Investigator CV	Mr Shakir Chowdhury	04 July 2013
Letter of invitation to participant	1	04 July 2013
Other: Email regarding funding		27 March 2012
Other: Dietary Plan	1	04 July 2013
Other: External Email	1	04 July 2013
Other: Internal Email	1	04 July 2013
Other: Letter regarding funding		04 July 2013
Other: Results letter to participants - screening	1	04 July 2013
Other: Results letter to participants - end of study	1	04 July 2013
Other: Signed page from academic supervisor		18 July 2013
Protocol	1	04 July 2013
Questionnaire: Food Frequency Questionnaire		
Questionnaire: International Physical Activity Questionnaire		
Questionnaire: Telephone Screening Questionnaire		
REC application	129696/477556/1/163	16 July 2013

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

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:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/YH/0253	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

pp DM Cattell

Dr Lynn Cawkwell
Chair

Email: nrescommittee.yorkandhumber-humberbridge@nhs.net

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments
"After ethical review – guidance for researchers"

Copy to: Dr Mario Siervo, Institute for Ageing and Health

Mr Andrew Johnston, The Newcastle upon Tyne Hospitals NHS Foundation Trust

NRES Committee Yorkshire & The Humber - Humber Bridge

Attendance at Committee meeting on 31 July 2013

Committee Members:

Name	*Profession*	*Present*	*Notes*
Reverend Annabel Barber	Lay Member	Yes	
Dr Stephen Beer	Consultant Physician	Yes	
Mrs Kate Bollington	Staff Nurse	Yes	
Dr Lynn Cawkwell	Senior Lecturer in Cancer Genetics	Yes	
Dr Fiona Cowdell	Senior Research Fellow	Yes	
Mr Michael Davidson	Retired Senior Personnel Manager	Yes	
Dr Karen Dunderdale	Chief Nurse	Yes	
Mr Michael Hockey	Consultant in Accident & Emergency	No	
Dr Sandeep Kapoor	Consultant Paediatrician	Yes	
Mrs Wendy Witter	Farm Administrator	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Diane Catterall	Coordinator

Appendix B. NO_3^- intake questionnaire

NITRATE INTAKE QUESTIONNAIRE

We are interested in finding out about the amount of nitrate containing food that people consume as part of their everyday lives. The questions will ask you about the types and frequency of food you consume in the **last 7 days**. Think about all the **foods you ate** in the **last 7 days**. Please answer each question or ask the investigators in this study if there are any questions that you are still unsure of.

Please tick on every line.

During the last 7 days how many times did you eat the following foods ?

Food Type	Frequency	Portion Size
a. Green leafy vegetables (Broccoli, cabbage, spinach, lettuce, etc).	☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	☐ Small ☐ Medium ☐ Large
b. Eggplant, Courgette, Turnip, Pumpkin.	☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	☐ Small ☐ Medium ☐ Large
c. Canned Products (Canned corn, canned peas, canned tomatoes, etc)	☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	☐ Small ☐ Medium ☐ Large
d. Cured Meat and/or Bacons.	☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	☐ Small ☐ Medium ☐ Large
e. Mature Cheese.	☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	☐ Small ☐ Medium ☐ Large
f. Beet and Beetroot	☐ Everyday ☐ 4 – 6 days ☐ 2 – 3 days ☐ 0 – 1 day	☐ Small ☐ Medium ☐ Large

Please tick every line

During the last 7 days how many times did you eat the following foods ?

FOOD TYPE	FREQUENCY			
	Everyday	4 – 6 days	2 – 3 days	0 – 1 day
1. Fresh Cheese				
• Brie	☐	☐	☐	☐
• Ricotta	☐	☐	☐	☐
• Cream Cheese	☐	☐	☐	☐
• Mozarella	☐	☐	☐	☐
• Others	☐	☐	☐	☐
2. Cereals				
• Potatoes	☐	☐	☐	☐
• Pasta	☐	☐	☐	☐
• Rice	☐	☐	☐	☐
• Breakfast Cereals	☐	☐	☐	☐
• Bread	☐	☐	☐	☐
• Savory Biscuits	☐	☐	☐	☐
3. Meat & Fish				
• Turkey	☐	☐	☐	☐
• Chicken	☐	☐	☐	☐
• Beef	☐	☐	☐	☐
• Pork	☐	☐	☐	☐
• Fish	☐	☐	☐	☐
• Other	☐	☐	☐	☐
4. Legumes				
• Bean	☐	☐	☐	☐
• Peas	☐	☐	☐	☐
• Chickpeas	☐	☐	☐	☐
• Lentils	☐	☐	☐	☐
5. Vegetables				
• Carrot	☐	☐	☐	☐
• Cucumber	☐	☐	☐	☐
• Pepper	☐	☐	☐	☐
• Tomato	☐	☐	☐	☐
• Mushroom	☐	☐	☐	☐
• Cauliflower	☐	☐	☐	☐

FOOD TYPE	FREQUENCY			
	Everyday	4 – 6 days	2 – 3 days	0 – 1 day
6. Fruits				
• Apples	☐	☐	☐	☐
• Banana	☐	☐	☐	☐
• Strawberries	☐	☐	☐	☐
• Peach	☐	☐	☐	☐
• Berries	☐	☐	☐	☐
• Others	☐	☐	☐	☐
7. Sweets				
• Chocolate	☐	☐	☐	☐
• Cakes	☐	☐	☐	☐
• Sweets	☐	☐	☐	☐
• Biscuits	☐	☐	☐	☐
• Ice Cream	☐	☐	☐	☐
8. Sauces				
• Ketchup	☐	☐	☐	☐
• Mayonnaise	☐	☐	☐	☐
• Others	☐	☐	☐	☐
9. Dairy Product				
• Milk	☐	☐	☐	☐
• Yogurt	☐	☐	☐	☐
10. Drink				
• Caffeinated	☐	☐	☐	☐
• Non – caffeinated	☐	☐	☐	☐
• Soft Drinks	☐	☐	☐	☐
• Fruit Juice	☐	☐	☐	☐

THANK YOU FOR TAKING YOUR TIME

TO COMPLETE THIS QUESTIONAIRE

Version 1, 24/9/2013

Appendix C. Protocols of laboratory analyses of inorganic NO_3^- and hyperglycaemia study

Protocol of 3', 5'-cyclic guanosine monophosphate (cGMP) assay

1. Plasma samples were thawed and kits reagent allowed equilibrating with room temperature before use.
2. Wash buffer (WB) prepared by adding 95 ml of deionised water (DW) to 5 ml of wash buffer concentrate provided by the kits' manufacturer.
3. Assay buffer 2 prepared by adding 50 ml of DW to 50 ml of the concentrated 2X assay buffer 2 supplied by the manufacturer.
4. Assay buffer 2 prepared in step 3 was used to dilute the standard stock provided by the manufacture (Table E.1).
5. Plasma samples diluted 1:10 using assay buffer 2

Table E.1: Preparation of the standards for cGMP Elisa kits

Standard	Assay buffer 2 in µL	Volume added of standard in µL	cGMP concentration (pmol/mL)
1	900	100 of stock solution	500
2	800	200 of standard 1	100
3	800	200 of standard 2	20
4	800	200 of standard 3	4
5	800	200 of standard 4	0.8

6. Standards, samples and reagents then added to the plate in the following order (Table E.2)

Table E.2: cGMP assay procedure and the addition of the reagents to the wells (volumes are in µL)

	Blank A1, B1	TA C1, D1	NSB E1, F1	Zero SD G1, H1	Standards A2-B3	Samples C3-H12
Assay buffer 2	-	-	100	100	-	-
Assay buffer 2	-	-	50	-	-	-
SD/Samples	-	-	-	-	100	100
Conjugate	-	-	50	50	50	50
Antibody	-	-	-	50	50	50
Seal the plate and incubate for 2 hours at room temperature						
Wash 3 times with WB (200µL) (empty the plate contents in the sink after each wash)						
Conjugate	-	5	-	-	-	-
pNpp substrate	200	200	200	200	200	200
Incubate for 1 hour						
Stop solution	50	50	50	50	50	50
Reading the plate with a plate reader at 405 nm optical density						

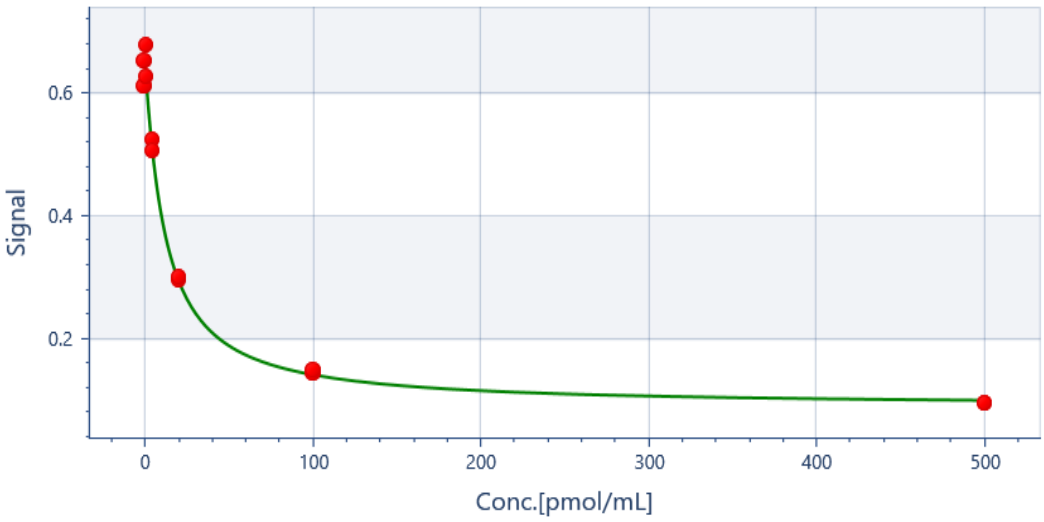


Figure E.1: Example standard curve of cGMP analysis

Protocol of 3 nitrotyrosine assay

1. Serum samples and reagents allowed to thaw and equilibrate with room temperature
2. I prepared 1X Wash Buffer by diluting the 20X WB (25 mL, provided by the manufacturer) in DW (475 mL).
3. I prepared 2X Incubation Buffer by adding 10 mL of 10X Blocking Buffer (provided by the manufacturer) to 40 mL of 1X Wash Buffer.
4. The diluted 2X HRP-Detector antibody prepared by adding 12 µL of the 1000X HRP-Detector antibody to 6mL of 2X Incubation Buffer (both provided by the manufacturer).
5. Standard prepared using standard stock and serial dilution was done using 1mL of 1X Wash Buffer. The following series of dilution were used to prepare the SDs (Table E.3).

Table E.3: Preparation of the standards for 3 nitrotyrosine Elisa kits

Standard	SD volume	Wash Buffer volume	Final volume	3 NT concentration (ng/mL)
1	600 µL of stock	0 µL	600 µL	2000
2	300 µL of 1	600 µL	900 µL	666.66
3	300 µL of 2	600 µL	900 µL	222.22
4	300 µL of 3	600 µL	900 µL	74.07
5	300 µL of 4	600 µL	900 µL	24.69
6	300 µL of 5	600 µL	900 µL	8.23
7	300 µL of 6	600 µL	900 µL	2.74
8	300 µL of 7	600 µL	900 µL	0.91

6. Standards, samples and reagents then added to the plate in the following order (Table E.4)

Table E.4: 3 nitrotyrosine assay procedure and the addition of the reagents to the wells

	Blank B3, B4	Standards A1-A4	Samples C3-H12
SD/Samples	-	50µL	50µL
HRP Detector antibody	50µL	50µL	50µL
Cover plate and incubate in room temperature for 2 hours			
Wash the plate 3 times using 300µL of WB, then contents emptied			
HRP Development Solution	100µL	100µL	100µL
Measure absorbance within 30 minutes using plate reader at 450 nm absorbance			

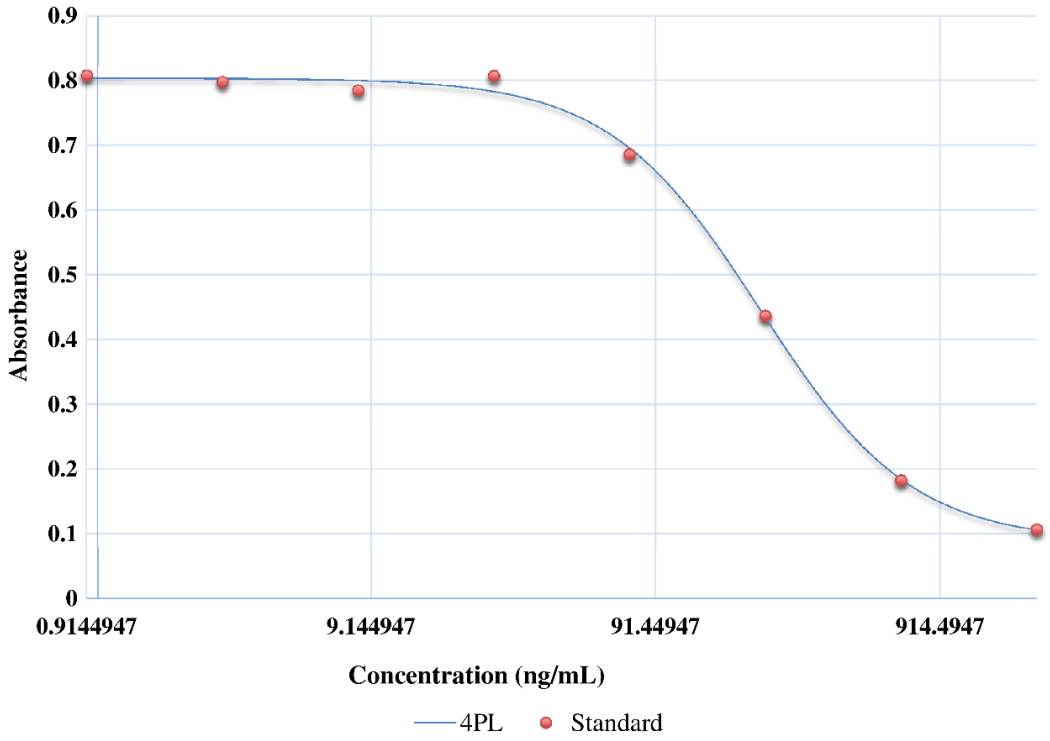


Figure E.2: Example standard curve of 3 nitrotyrosine analysis

Protocol of Human vascular injury multiplex (P selectin, E selectin, intercellular adhesion molecule 3 [ICAM 3] and thrombomodulin) assay

1. Preparation of Blocker A: adding 20 mL of DW to 1.25 g Blocker A powder. Stir the mixture until all protein is suspended then add 5 mL of MSD Phosphate Buffer (provided with the kit).
2. I added 150 µL Blocker A to each well of the plate. Sealed the plate and incubated it on a plate shaker (600-700 rpm) at room temperature for 1 hour.
3. Preparation of the washing solution: the washing solution composed of phosphate buffered saline with 0.05% Tween-20 (PBS-T). I added 1 PBS tablet to 1 L of deionised water. After complete disintegration of the tablet, I added 0.5 mL of Tween-20.
4. Washing of the plates: after 1 hour incubation with Blocker A, I washed the plate 3 times with 200 µL per well of the PBS-T.
5. Preparation of standards (Table E.5): to prepare the eight serial dilutions of the calibrators (standards), I used the Diluent 10 and a vial of Human Vascular Injury I Calibrator Blend (both provided with the kits). I started with Human Vascular Injury I Calibrator Blend as the top of the curve (1000 ng/mL). The second standard (250 ng/mL) composed of 20 µL of the stock added to 60 µL of Diluent 10. The other five standards prepared by doing a 4-fold serial dilution to make calibrator solutions of 63, 16, 3.9, 0.98 and 0.24 ng/mL. The 8th calibrator is diluent 10 alone (zero calibrator).

Table E.5: Preparation of the standards for the human vascular injury I Elisa kits

Standard	SD volume	Assay buffer volume	Final volume	Biomarkers concentration (ng/mL)
Stock	N/A	N/A	N/A	1000
1	20 µL of stock	60 µL	80 µL	250
2	20 µL of 1	60 µL	80 µL	63
3	20 µL of 2	60 µL	80 µL	16
4	20 µL of 3	60 µL	80 µL	3.9
5	20 µL of 4	60 µL	80 µL	0.98
6	20 µL of 5	60 µL	80 µL	0.24
7 (Blank)	N/A	80 µL	80 µL	0

6. After complete washing of the plate, I added 40 µL of diluent 10 to each well of the plate.
7. I added 10 µL of Calibrator (start with the highest concentration 1000 ng/mL) in duplicate to the wells from A1 to H2.
8. 10 µL of samples then added to wells A3 to H12 in duplicates. The plate then incubated for 2 hours on shaker (700 rpm) at room temperature.
9. After 2 hours incubation, I washed the plate three times with 200 µL of PBS-T.
10. Preparation of 1X detection antibody: I added 60 µL of 50X SULFO-TAG Detection Antibody Blend (provided with kit) to 2.94 mL of Diluent 10.

11. I added the 25 µL of the detection antibody solution to each well of the plates and incubate for another 1 hour at room temperature with shaking at 700 rpm.
12. Preparation of the read buffer: I added 5 mL of 4X Read Buffer T (provided with kit) to 15 mL deionised water
13. One hour later, I washed the plate 3 times again with PBS-T (200 µL).
14. I prepared the MSD reader so the plate can be read immediately after adding the read buffer to the plates (150 µL).

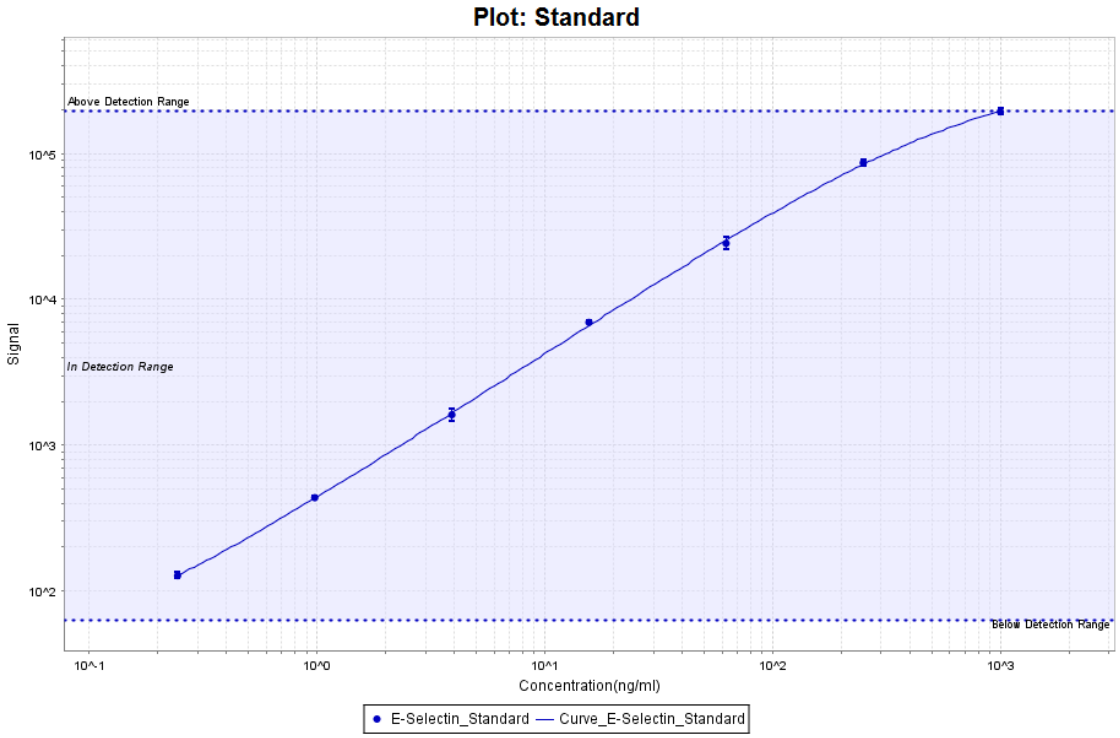


Figure E.3: Example standard curve of E selectin analysis

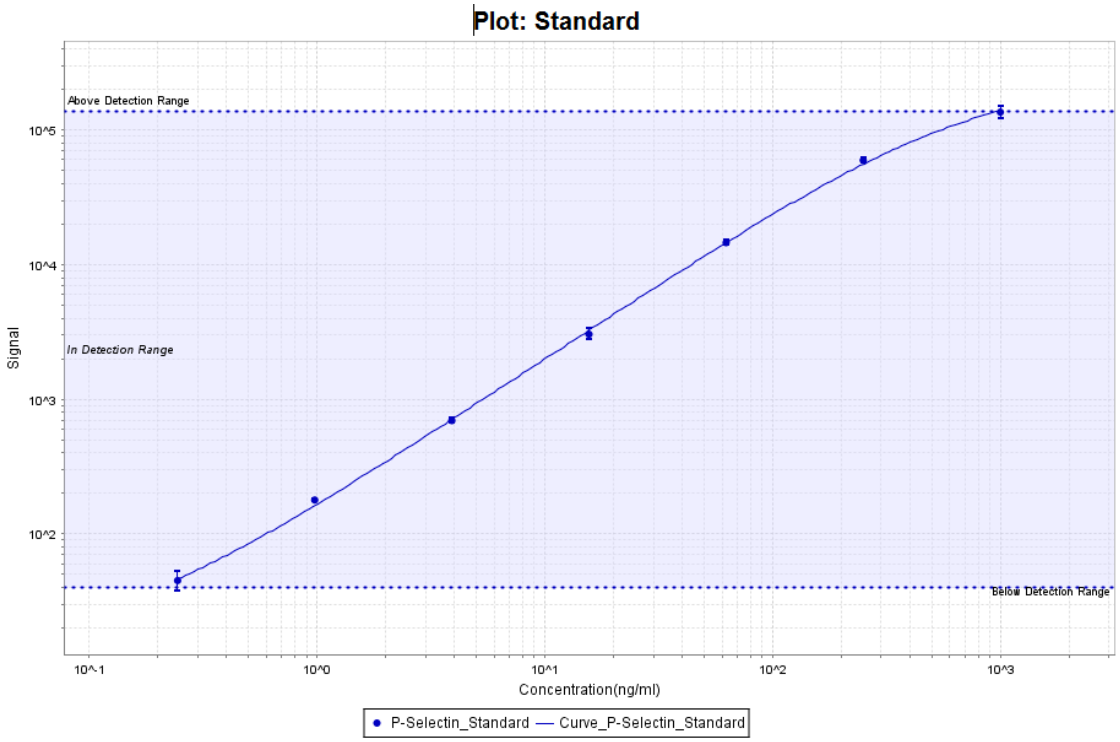


Figure E.4: Example standard curve of P selectin analysis

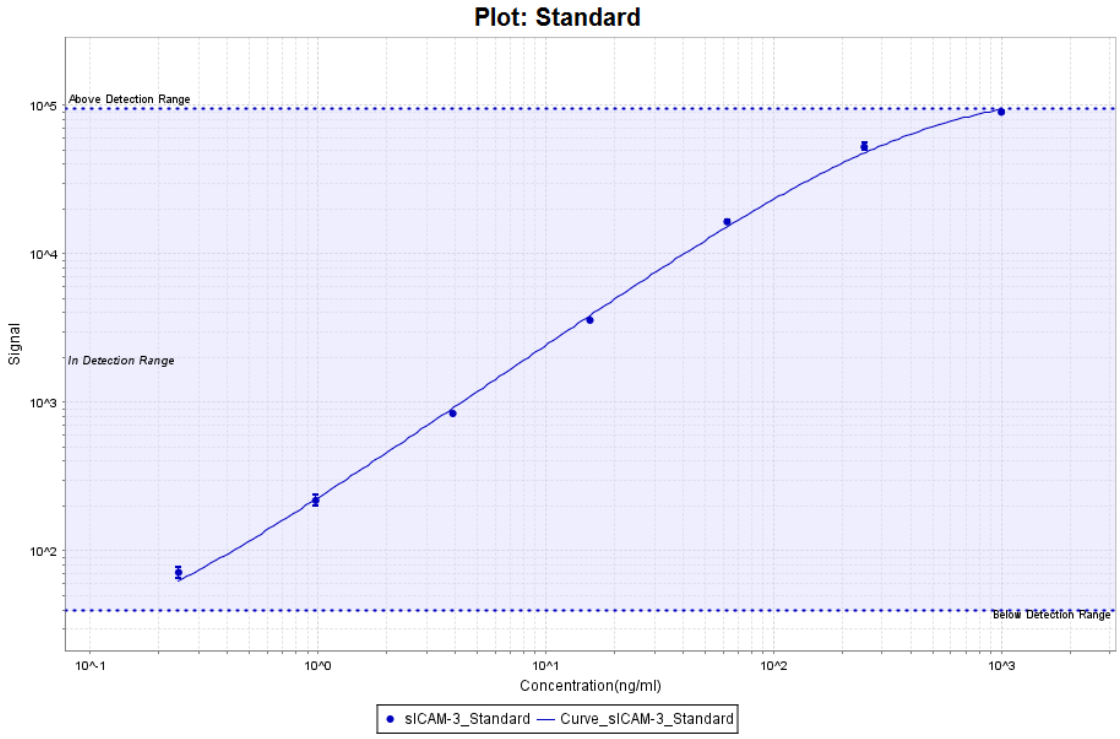


Figure E.5: Example standard curve of intercellular adhesion molecule 3 (ICAM-3) analysis

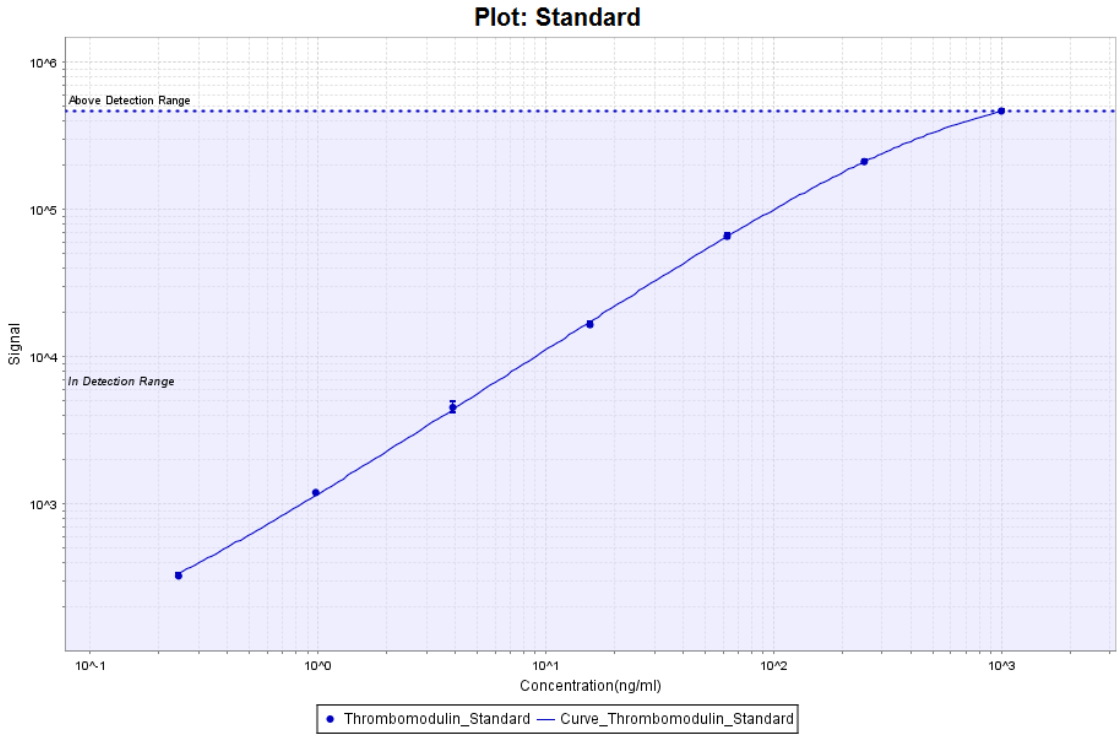


Figure E.6: Example standard curve of thrombomodulin analysis

Appendix D. Ethical approval of the inorganic NO_3^- and vitamin C study

EoSRES

NHS SCOTLAND
Research Ethics Service

East of Scotland Research Ethics Service (EoSRES) REC 2
Tayside Medical Sciences Centre (TASC)
Residency Block C, Level 3
Ninewells Hospital & Medical School
George Pirie Way
Dundee DD1 9SY

Mr Ammar Ashor
PhD Student
Institute for Ageing and Health
Campus for Ageing and Vitality, Newcastle University
Newcastle Upon Tyne
NE4 5PL

Date: 23 April 2014
Your Ref:
Our Ref: LR/**DL/14/ES/0059**
Enquiries to: Mrs Lorraine Reilly
Extension: Ninewells extension: 83878
Direct Line: 01382 383878
Email: eosres.tayside@nhs.net

Dear Mr Ashor

Study title: **Acute effects of dietary nitrate and vitamin C supplementation on blood pressure and endothelial function in young and older human subjects: a 2*2 factorial cross-over trial**
REC reference: **14/ES/0059**
IRAS project ID: **139405**

The Proportionate Review Sub-committee of the East of Scotland Research Ethics Service REC 2 reviewed the above application on 22 April 2014.

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 Assistant Co-ordinator Mrs Diane Leonard, Diane.Leonard@nhs.net.

Ethical opinion

No ethical issues noted.

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

POSITIVE ABOUT DISABLED PEOPLE

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved were:

Document	Version	Date
Advertisement	2	18 March 2014
Covering Letter		10 April 2014
Evidence of insurance or indemnity		26 July 2013
Investigator CV		
Letter from Sponsor		26 March 2013
Letter of invitation to participant	2	18 March 2014
Other: CV - Dr Mario Siervo		04 July 2013

Document	Version	Date
Other: CV - Professor John Mathers		08 March 2012
Other: Letter from Funder		02 February 2014
Other: Result letter to participants at end of study	2	19 February 2014
Other: BNF award letter		13 March 2013
Other: 2-Day standardised diet	1	24 September 2013
Other: Leaflet	2	18 March 2014
Other: Email recruitment poster	2	18 March 2014
Participant Consent Form	2	19 February 2014
Participant Information Sheet	3	18 March 2014
Participant Information Sheet: 2	3	19 February 2014
Protocol	2	25 September 2013
Questionnaire: Food Frequency		
Questionnaire: International Physical Activity		
Questionnaire: Nitrate Intake		
Questionnaire: Screening	1	24 September 2013
REC application: 139405/594144/1/624		04 April 2014

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

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:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

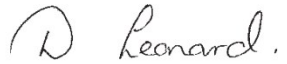
The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.
information is available at National Research Ethics Service website > After Review

14/ES/0059	Please quote this number on all correspondence

Yours sincerely



for Dr Anthony Davis
Vice-Chair

E-mail: eosres.tayside@nhs.net

Enclosures: List of names and professions of members who took part in the review "After ethical review – guidance for researchers"

Copy to: Andrew Johnston, Newcastle Upon Tyne Hospitals NHS Foundation Trust

East of Scotland Research Ethics Service REC 2

Attendance at PRS Sub-Committee of the REC meeting on 22 April 2014

Written comments received from:

Name	Position
Dr Anthony Davis	Vice-Chair, Consultant Anaesthetist
Mr Dougie McPhail	Primary Care Development Pharmacist
Mrs Gail Watson	Senior Clinical Research Associate (SCRA)

Also in attendance:

Name	Position (or reason for attending)
Mrs Diane Leonard	Assistant REC Co-ordinator

Appendix E. 2-Day Standardized (low vitamin C and 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 vitamin C and 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 for you.
A list of **food allowed and food to avoid** is shown below together with some examples of low nitrate meals.

FOOD TO AVOID		FOOD ALLOWED	
• Arugula	• Fresh Pepper	• Apples	• Peas
• Bacon	• Grapefruit	• Apricots	• Pineapples
• Bananas	• Green Beans	• Artichoke	• Plums
• Basil	• Ketchup	• Asparagus	• Potatoes
• Beans	• kiwi	• Avocados	• Poultry
• Beans (French)	• Leek	• Barley	• Prickly pears
• Beans (Runner)	• Lemon	• Beans (Broad)	• Quinces
• Beet	• Lettuce	• Beansprout	• Rice
• Beetroot	• Mandarin	• Blueberries	• Ricotta
• Blackberries	• Mandarin	• Blue Cheese	• Soybean oil
• Broccoli	• Oranges	• Bread	• Sweet corn
• Brussels sprouts	• Mango	• Butter	• Sweet potato
• Cabbage	• Meat Cured	• Camembert	• Watermelon
• Canned asparagus	• Melon	• Cantaloupe	• Wheat
• Canned beans	• Mustard and cress	• Cereal	• Wheat flour
• Canned corn	• Onion (Spring)	• Cheese Soft	• White bread
• Canned peas	• Orange	• Cheese Spread	• Winter melon
• Canned pumpkin	• Papaya	• Cherries	• Yogurt
• Canned spinach	• Parsley	• Chicken	
• Canned tomatoes	• Peanuts	• Corn	
• Carrot	• Pumpkin	• Corn oil	
• Cauliflower	• Radish	• Cream of Wheat	
• Celery	• Raspberries	• Egg	
• Chard	• Red and yellow peppers	• Eggplant	
• Chicory	• Rhubarb	• Feta Cheese	
• Chinese Leaf	• Rocket	• Flour	
• Chives	• Shellfish	• Fresh Cheese	
• Clementine	• Spinach	• Fresh Sausage	
• Coriander	• Strawberries, blueberries	• Fresh Tomato	
• Corned Beef	• String beans	• Garlic	
• Courgette	• Summer squash	• Grapes	
• Cucumber	• Sunflower oil	• Mayonnaise	
• Cured Beef	• Sweet almond oil	• Meat Fresh	
• Cured Pork	• Turnip	• Milk	
• Cured Salami	• Watercress	• Minced Meat	
• Cured Turkey	• Wheat germ oil	• Mushrooms	
• Currants		• Oat Meal	
• Dill		• Olive oil	
• Dried beef		• Onion	
• Endive		• Parsnip	
• Fennel		• Pasta	
• Frankfurt		• Peach	
• Ham			
• Hot peppers			
• Kale			

We would also like to remind you that while you are on the study you should try to drink and cook with the same **water**. Different sources of water may contain different amount of nitrate and in this way we will ensure that the amount of nitrate you will be consuming will be constant during the week.

Drinks

- Consumption of coffee and tea is allowed during the study. However, we will ask to drink the same amount you usually drink and to not change these habits during the study.
- We will ask you to consume alcoholic drinks with moderation and not to have more than one alcoholic drink per day.
- We would like to ask you to consume soft-drinks in moderation and not to have more than one can of drinks (330ml) per day.
- High energy, caffeinated drinks (for example: red bull) are not allowed during the study.

Some examples of meals that you could have are showed below:

Breakfast
• Tea, coffee
• Milk with corn flakes or special K
• Butter on toast
• Plain croissant, chocolate muffin
• Plain Yogurt
• Jam (excluding strawberry, orange, lemon)
• Scrambled eggs
Lunch
• Egg mayo sandwich
• Chicken sandwich
• Panini with mozzarella and fresh tomato
• Chicken fried rice
• Fresh meat or fish (grilled, steamed or fried)
• Potatoes (fried, roasted or boiled)
• Baked pasta with chicken and mozzarella
• Mushroom and chicken risotto
• Chocolate bar
• Chocolate cake, apple crumble
• Apple, apricot, pear
Dinner
• Grilled meat (beef, chicken, pork) with potatoes and mashed peas
• Pasta with cream cheese, mushrooms and chicken
• Grilled fresh sausages with mashed potatoes
• Omelette, mushrooms and potatoes
• Pizza with fresh tomato and mozzarella
• Cheeseburger with chips (no ketchup or mayonnaise is allowed)
• Fish and chips
• Apple crumble with custard
• Chocolate cake
• Apple, apricot, pear

Appendix F. Protocols of laboratory analyses of inorganic NO_3^- and vitamin C study

Procedure of the NO_3^- and NO_2^- analyses

a. Procedure of the analyses:

1. Deproteinisation of the samples: I added methanol in 1:1 volume (1.25mL of Methanol to 1.25mL of sample) and vortex directly after mixing. Then, centrifuge the mixture for 10 minutes (13000RPM). I collected the supernatant and left the precipitants.
2. I_3 solution Preparation: I added 2g of Potassium iodide (KI) to 1.3g Iodine in 40 mL Deionised Water (DW), these should be dissolved thoroughly. Then, I added 140 mL of acetic acid to the mixture. 10-15mL of I_3 product then injected to the purge vessel.

- Preparation of vanadium chloride: The first step was to prepare 1N/1M HCl by adding 82.5mL of 36% HCl to 1 litre of DW. The second step adding 0.4g of vanadium (III) chloride (MW 157.3) to 50mL of 1 N of HCl to have 50.9mM of vanadium chloride. I mixed thoroughly the product in light-protected container then filtered it through filter paper.

3. I added 200µL of antifoam B emulsion (aqueous-silicone emulsion, Sigma-Aldrich). This emulsion contain nitrite, therefore we should wait for few minutes until it return back to baseline.
4. Preparation of NAOH: I added 20g of NAOH to 0.5mL of DW to make 1 Mol of NAOH (this NAOH is interposed between the purge vessel and the chemiluminescence analyser; it acts as a chemical trap of the acid produced from the reaction in the purge vessel).
5. Preparation of standards:

- I added 345mg of Sodium Nitrite into 50mL of DW. This will give 100mM of $NaNO_2$.
- To prepare 1 mM of $NaNO_2$ (1/100 Dilution). I took 0.5mL of the 100mM stock and added it to 49.5mL of DW.
- Preparing 1uL of stock solution: from 50mL DW, replace 50µL of that DW with 50µL of 1mM stock.
- I thoroughly clean the injection syringe before use. Then, I injected 200µL of DW in the vessel. The peaks developed by the DW, should be subtracted from the measurements.
- Standard Curve: I injected serial volumes (50mL, 100mL, 150mL and 200mL) of 1µL stocks (in duplicates) to produce NO_3^-/NO_2^- standard curves.

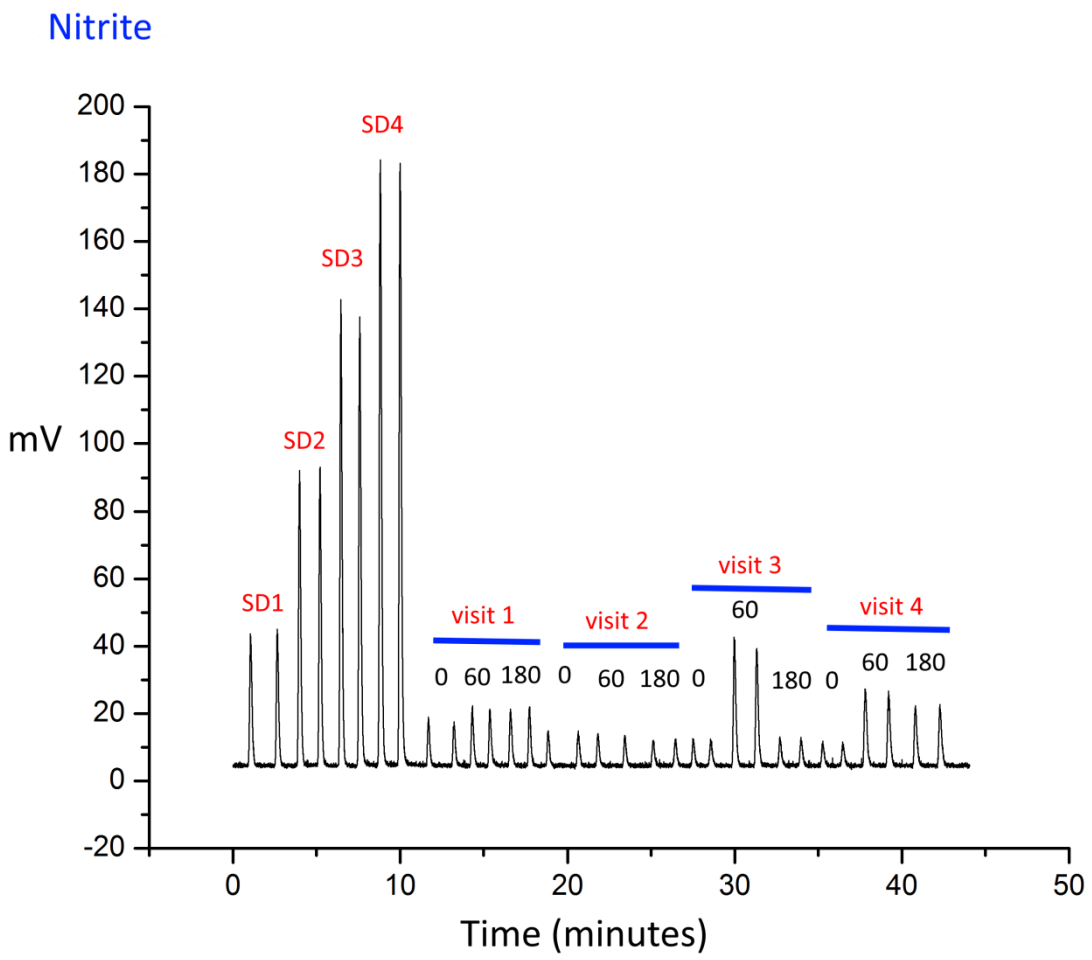


Figure F.1: Example of traces produced from injecting standard well-known concentrations of NO_2^- and plasma samples into Sievers gas-phase chemiluminescence nitric oxide analyser. SDs represent standards and the 0, 60, 180 represent the selected study time points analysed.

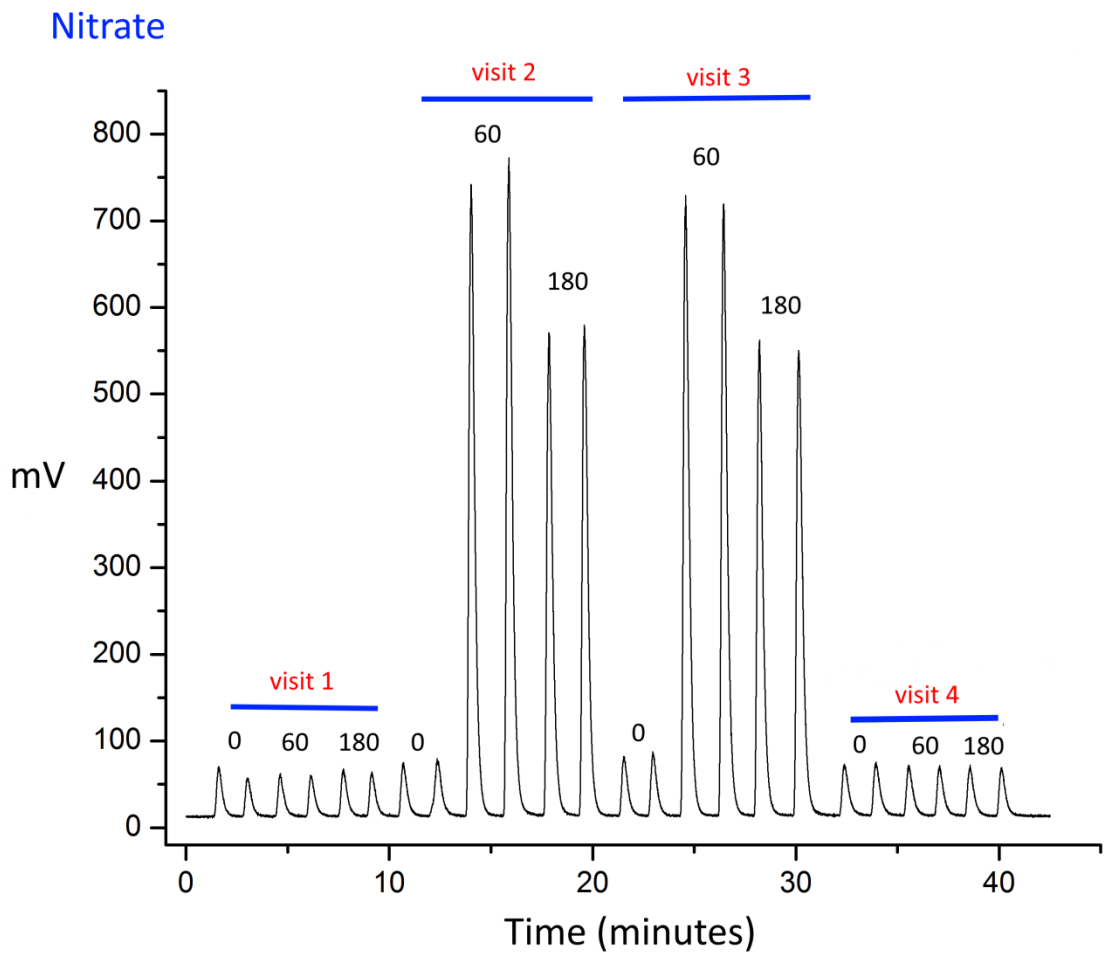


Figure F.2: Example of traces produced from injecting plasma samples into Sievers gas-phase chemiluminescence nitric oxide analyser. 0, 60, 180 represent the selected study time points analysed.

Protocol of vitamin C analysis

- Protocol of analysis:

1. Preparation of the Buffer (the mobile phase): I prepared 100 µM of Ammonium Acetate by adding 7.708g of Ammonium Acetate (Sigma-Aldrich) to 1L of DW. I stirred the product for 5 minutes until complete dissolution of the particles, then I checked the pH of the solution (it should be 6.8). Then I took 300 mL of Ammonium Acetate and add them to 700 mL of acetonitrile (30:70). I filtered the mixture before using in the HPLC.
2. Preparation of MPA: I added 5g of MPA to 50mL of DW to obtain (10% MPA).
3. Preparation of standards: Vitamin C stock (0.5mg/mL) prepared by adding 50mg of ascorbic acid to 100mL of DW (500mg/L). I took 10mL of ascorbic acid solution and added it to 90mL of DW.

Table F.1: Preparation of the standards for vitamin C analysis. SD: standard; DW: deionised water; MPA: meta-phosphoric acid.

Standards	Concentration	Volume of stock	Volume of MPA	Volume of DW
SD1	0.01mg/mL	200µL	250µL	550µL
SD2	0.008mg/mL	160µL	250µL	590µL
SD3	0.006mg/mL	120µL	250µL	630µL
SD4	0.004mg/mL	80µL	250µL	670µL
SD5	0.002mg/mL	40µL	250µL	710µL
Blank	0.0mg/mL	0	250µL	750µL

4. Preparation of the samples (deproteinisation): I added 200 µL of plasma sample to 200 µL of MPA (10%) and left the product for 5 minutes on ice. I centrifuged the samples for 5 minutes at 14000rpm. The last step is to dilute 100µL of the supernatant with 100µL of DW.

Assay procedure of dehydroascorbic acid (DHA)

- Assay procedure:

1. Samples preparation: I added 100µL of serum sample to 1mL of Assay Buffer (provided by the manufacturer) on ice. I centrifuged the product (16000g, 4ºC) for 20 minutes. I took the supernatant and kept it on ice for further analysis.

2. Standard preparation: first step was to reconstitute the standard stock (10µL) provided by the manufacturer with 2.861 mL of DW. Then I added 0.9mL of DW to 0.1mL of the reconstituted standard to obtain 100µL of DHA standard.
3. I added 20µL of standards and samples to the allocated wells. Then, I added Reagent I (160µL) and Reagent II (20µL) (provided by manufacturer) to each well. I measured then with Plate Reader at 265nm and recorded the absorbance at 10 and 130 seconds.
4. The following equation then used to calculate the concentration of DHA in serum samples:

DHA (µmol/mL) = 0.1 × [OD sample (130s) - (OD sample (10s)]/ [OD standard (130s) - (OD standard (10s)]/volume of sample

OD: optical density

Tetrahydrobiopterin assay procedure

- Assay procedure

1. Sample preparation: I diluted 10µL of plasma samples with 385µL sample diluent (provided by the manufacturer) OR
2. Prepare wash buffer: I added 20mL of wash buffer concentrate (provided by the manufacturer) to 480mL of DW
3. Prepare of standards (Table F.2): I centrifuged the standard vial (provided by the manufacturer) at 6000-10000rpm for 30s. I reconstituted the standard with 1mL of sample diluent (provided by the manufacturer). Figure F.3 showing an example of standard curve of BH4 analysis.
4. Preparation of HRP-conjugate: I centrifuged the stock provide by the manufacturer then diluted 10µL of HRP-conjugate with 990µL of HRP-conjugate diluent.
5. Assay procedure are summarised in Table 5.3 below.

Table F.2: Preparation of the standards for tetrahydrobiopterin (BH4) analysis

Standard	Sample diluent	Volume of stock added	BH4 concentration Pg/mL
6	250µL	250µL of stock solution	50
5	250µL	250µL of SD6	25
4	250µL	250µL of SD5	12.5
3	250µL	250µL of SD4	6.25
2	250µL	250µL of SD3	3.12
1	250µL	250µL of SD2	1.56

. SD: standard.

Table F.3: Steps followed in tetrahydrobiopterin (BH4) analysis.

	Blank G12-H12	Standards A1-D2	Samples E2-F12
Standards and samples		50µL	50µL
HRP-conjugate		50µL	50µL
Covered with adhesive film and incubate for 1h at 37ºC			
Wash 5x the plate using 250µL of wash buffer			
substrate	100µL	100µL	100µL
Incubate for 15-20 min at 37ºC			
Stop solution	50µL	50µL	50µL
Read the plate at 450nm			

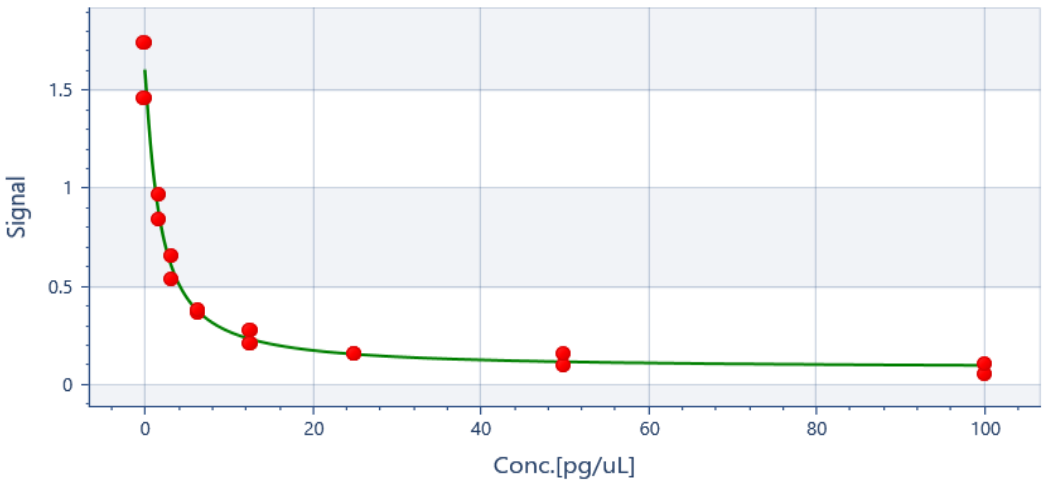


Figure F.3: Example standard curve of tetrahydrobiopterin (BH4) analysis

Protocol of 3 nitrotyrosine assay

a. Protocol of the assay:

7. Serum samples and reagents allowed to thaw and equilibrate with room temperature

8. 1X WB prepared by diluting the 10X WB (provided by the manufacturer) in DW.

9. The diluted anti-nitrotyrosine antibody prepared by adding the anti-nitrotyrosine (HRP-conjugated) detection antibody concentrate (1:100) to Nitrotyrosine Antibody Diluent (both provided by the manufacturer and the dilution factor 1:100).

10. Standard prepared using standard stock and serial dilution was done using Sample and Standard Diluent (both provided by the manufacturer). The following series of dilution were used to prepare the SDs (Table 5.4).

Table F.4: Preparation of the standards for 3 nitrotyrosine Elisa kits

Standard	SD volume	Assay buffer volume	Final volume	3 NT concentration (nmol/mL)
Stock	N/A	N/A	N/A	408 µM
1	10 µL of stock	500 µL	250 µL	8000
2	250 µL of 1	250 µL	250 µL	4000
3	250 µL of 2	250 µL	250 µL	2000
4	250 µL of 3	250 µL	250 µL	1000
5	250 µL of 4	250 µL	250 µL	500
6	250 µL of 5	250 µL	250 µL	250
7	250 µL of 6	250 µL	250 µL	125
8	250 µL of 7	250 µL	250 µL	62.5
9 (Blank)	N/A	250 µL	250 µL	0

11. Standards, samples and reagents then added to the plate in the following order (Table F.5). Figure F.4 showing an example of 3 nitrotyrosine standard curve.

Table F.5: 3 nitrotyrosine assay procedure and the addition of the reagents to the wells

	Blank B3, B4	Standards A1-A4	Samples C3-H12
SD/Samples	-	50µL	50µL
HRP-conjugate antibody	-	50µL	50µL
Standard and sample diluent	50µL	-	-
Antibody diluent	50µL	-	-
Cover plate and incubate in room temperature for 1 hour			
Wash the plate 3 times using 300µL of WB, then contents emptied			
TMB substrate	100µL	100µL	100µL
Cover plate and incubate in the dark, at room temperature, for 30 minutes			
Stop solution	100µL	100µL	100µL
Measure absorbance within 30 minutes using plate reader at 450 nm absorbance			

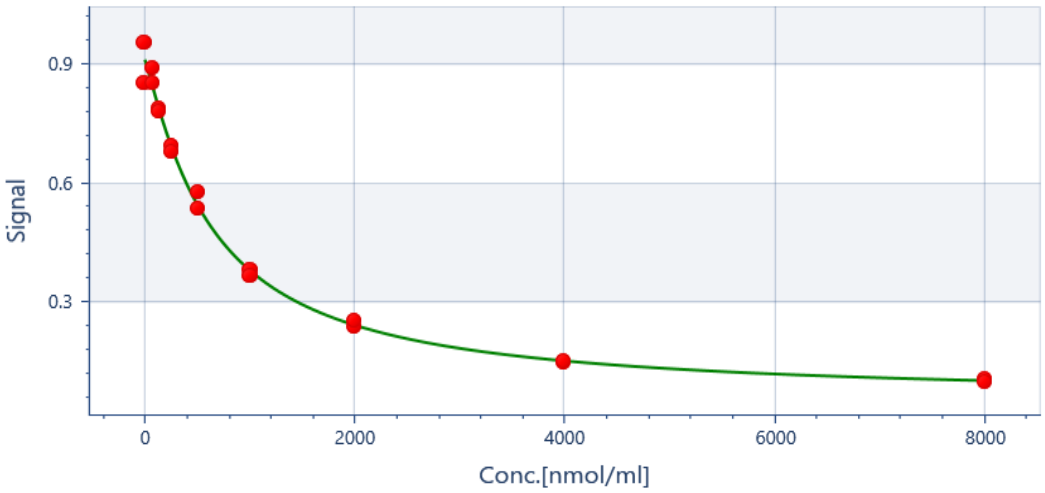


Figure F.4: Example of standard curve of 3 nitrotyrosine analysis

Appendix G. Chapter 5 supplementary figures

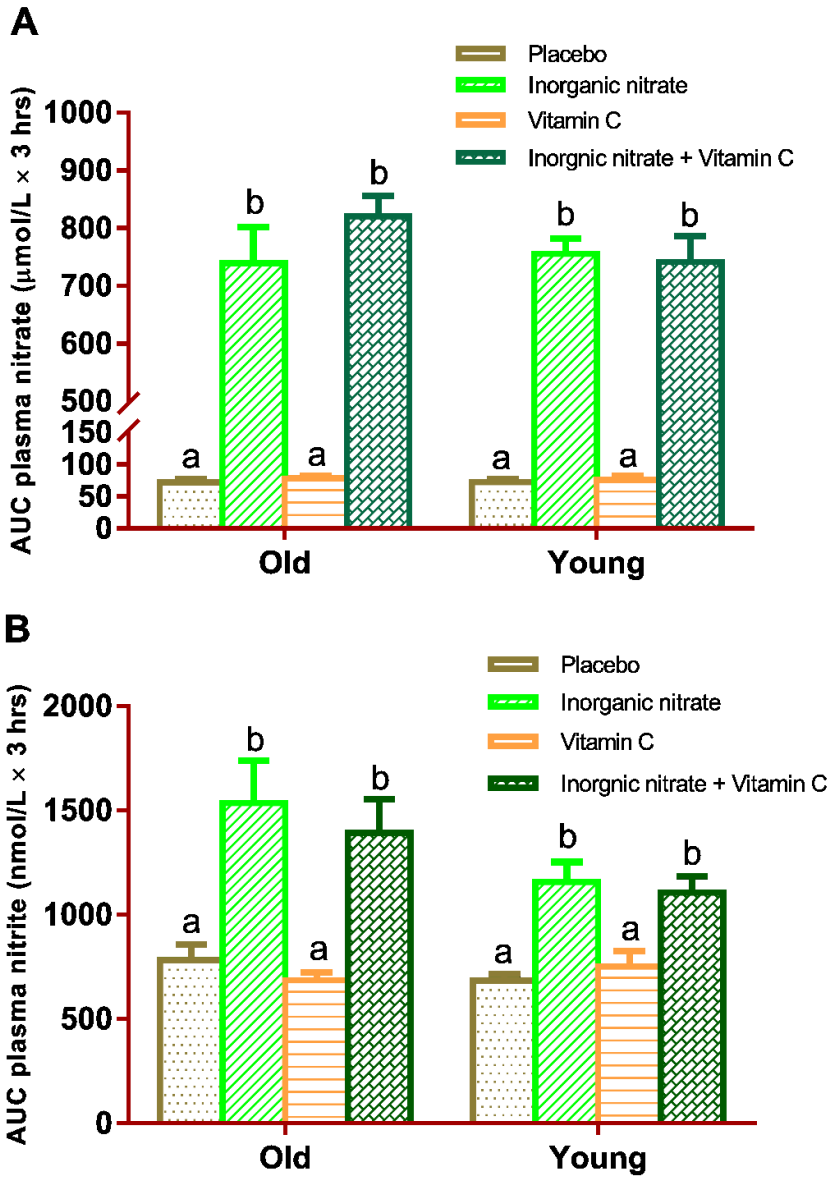


Figure 1: Plasma NO_3^- (A) and NO_2^- (B) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo ($P < 0.05$). AUC: 3 hours area under curve.

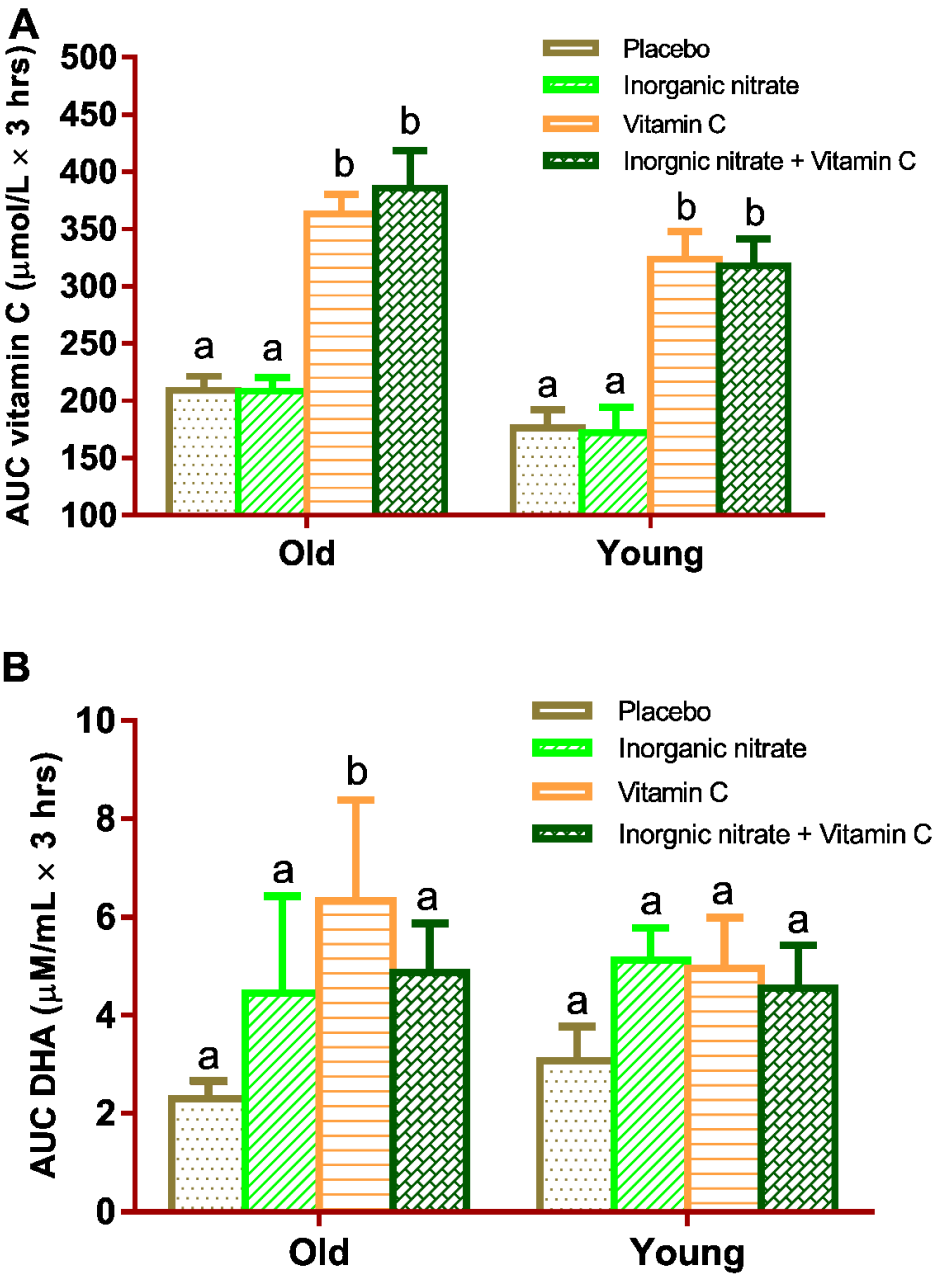


Figure 2: Plasma vitamin C (A) and dehydroascorbic acid (DHA) (B) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). *: P < 0.05. AUC: 3 hours area under curve.

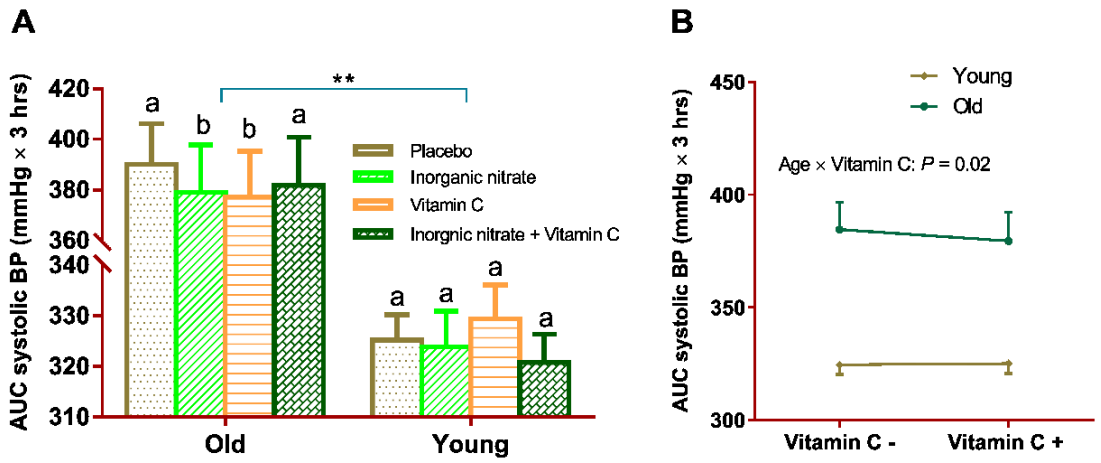


Figure 3: Systolic blood pressure (BP) (A) and the age × vitamin C interaction (B) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo ($P < 0.05$). **: $P < 0.01$. AUC: 3 hours area under curve.

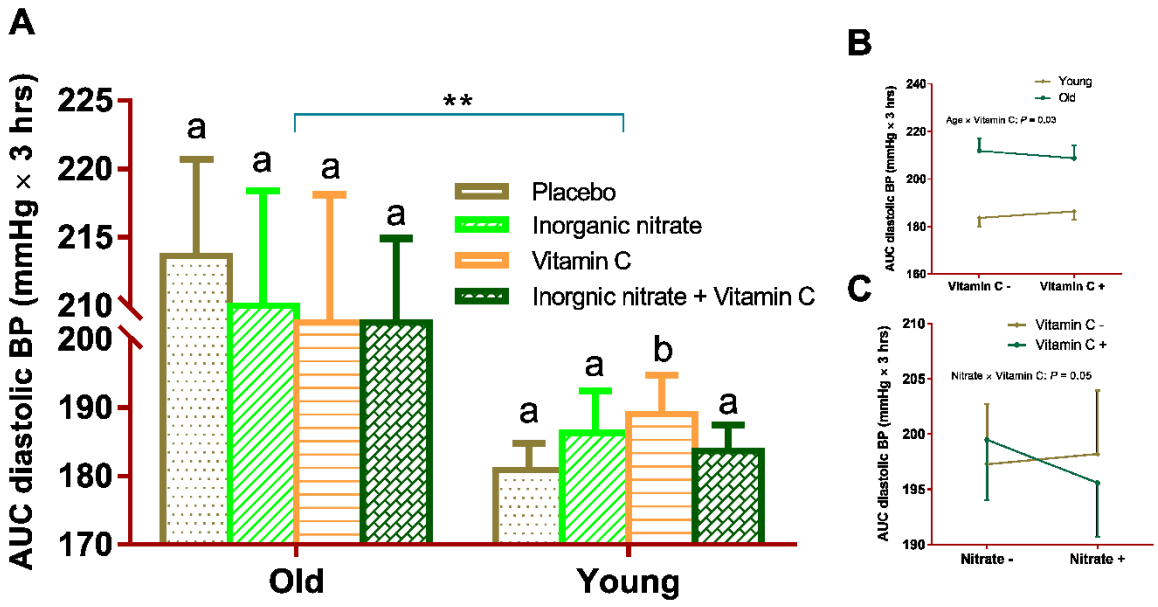


Figure 4: Diastolic blood pressure (BP) (A), the age × vitamin C interaction (B) and inorganic NO_3^- × vitamin C interaction (C) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). **: P < 0.01. AUC: 3 hours area under curve.

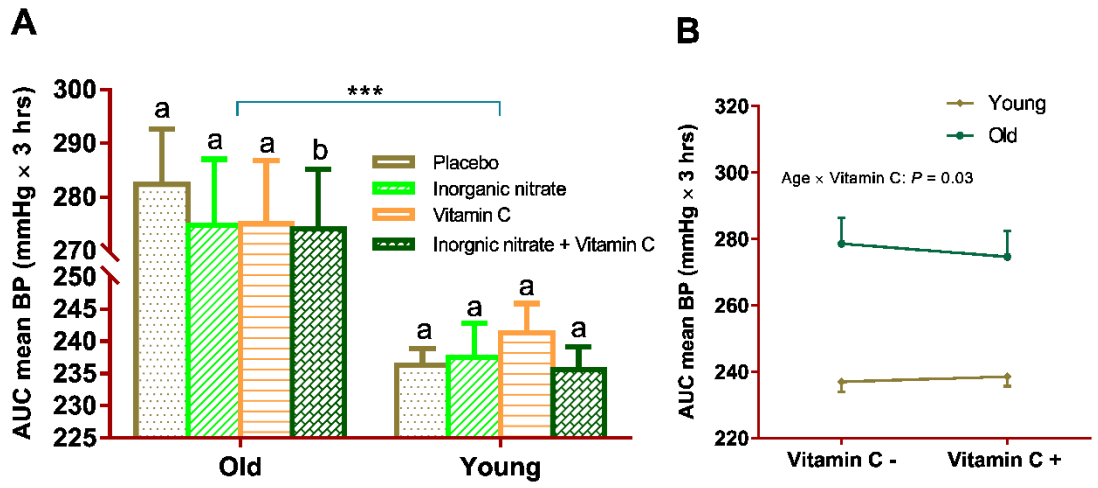


Figure 5: Mean arterial blood pressure (BP) (A), the age × vitamin C interaction (B) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). ***: P < 0.001. AUC: 3 hours area under curve.

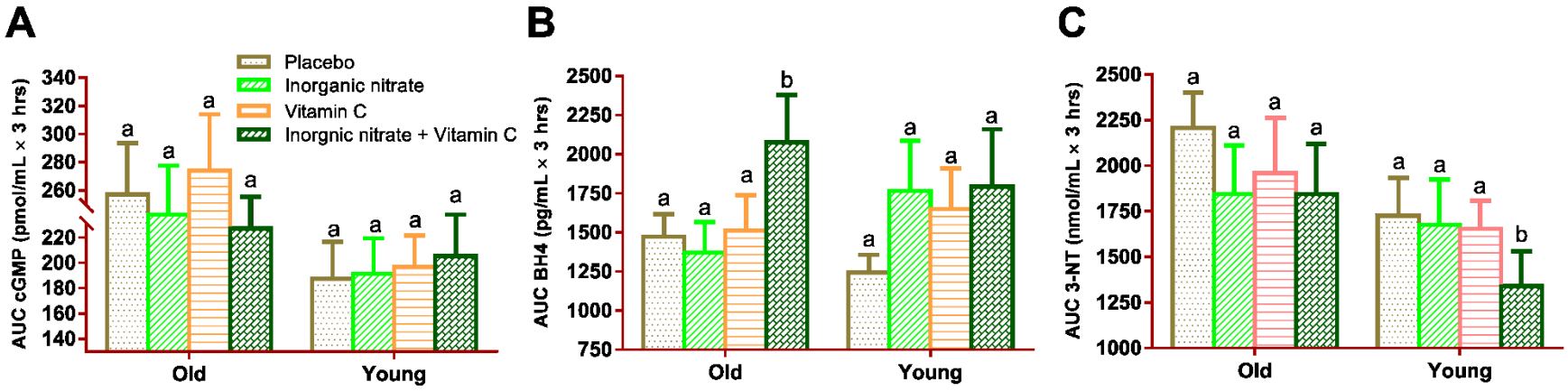


Figure 6: Plasma cyclic guanosine monophosphate (cGMP) (A) tetrahydrobiopterin (BH4) (B) and 3 nitrotyrosine (3-NT) (C) in younger (n= 10) and older participants (n= 10) given a single dose of inorganic NO_3^- (7 mg/kg body weight), vitamin C (20 mg/kg) both agents combined or their placebos in a 2×2 factorial crossover design. Values are means ± SEMs. Data were analysed using linear mixed model. Unmatched letters denote significantly different from placebo (P < 0.05). AUC: 3 hours area under curve.

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