University of Reading

Impacts of Whole Grain Oats intake on Blood Pressure and Vascular Function; the role of Phenolic Acids on the Renin Angiotensin System

Thesis submitted for the Degree of

Doctor of Philosophy

Department of Food and Nutritional Sciences

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August 2018

Declaration

I confirm that this is my own work and that the use of all material from other sources has been properly and fully acknowledged.

Sarah Al Sharif

Reading, August 2018

Acknowledgements

First of all, I would like to thank Allah for giving me the strength and courage to do my research and complete this thesis.

I would like to thank Professor Jeremy Spencer for giving me the opportunity to complete my PhD under his supervision, keeping me motivated throughout the writing and editing of my thesis. Special thanks to my second supervisor Dr Gunter Kuhnle for his invaluable advice and feedback on my research. Also, I gratefully acknowledge the funding received towards my PhD from king abdulaziz university, Jeddah Saudi Arabia.

My deep appreciation goes out to Dr. Gessica Serra for continues encouragement and advice through the challenges that faced me during the laboratory work. This PhD study would not have been possible without the corporation and support by Dr.Manuel Y. Schär, Gulten Soycan and Angelika Kristek throughout both clinical trials. I would also like to thank Dr Michelle Weech for always being available to give advices and information about LDI analysis. Also, thank all the staff in the Hugh Sinclair Unit, for all the advice, support in guiding me through the clinical trials.

I would like to thank my family and close friends, your unconditional love, support, patience, optimism and trust was more valuable than you could ever imagine. My mom, I am really lucky to have you, thank you for creating various opportunities in life for me. My grandmother, sisters and brothers, thank you for always believing in me. A very special word of thanks goes for my Fiancé Mohammed whose faithful support during the final stages of this Ph.D.

Abstract

Hypertension is a major risk factor for cardiovascular disease and there is substantial evidence that its reduction towards the normotensive state significantly reduces the risk of developing cardiovascular morbidity and mortality. In addition to pharmacological treatment of hypertension, diet is also capable of counteracting the development of high blood pressure and reducing it from an elevated state. Diets rich in plant foods have been found to attenuate blood pressure rises over time and a number of polyphenol-rich foods/beverages derived from plants have been shown to induce beneficial effects on endothelial function and blood pressure in human clinical trials. Polyphenol rich foods include many fruits and vegetables, cocoa, tea, coffee and their derived extracts. Less is known regarding other polyphenol-rich staple foods, such as whole grains which also contain relatively high levels of these bioactives. Whole grain oats are a rich source of small phenolic compounds, such as ferulic acid and avenanthramides, in addition to their widely recognised fibre content, so may also be capable of beneficial changes to endothelial function and blood pressure. This thesis tests this hypothesis and attempts to understand the actions of small phenolics and their metabolites from oats on the renin-angiotensin aldosterone system (RAAS), a major regulator of human blood pressure homeostasis.

To investigate the actions of the most abundant oat phenolics on the RAAS, we utilised kidney juxtaglomerular and HUVEC cells to test whether they and their metabolites influenced renin expression and whether this was regulated via interactions with the ERK-CRB/ATF pathway. Renin gene expression was significantly decreased by exposure to several polyphenols, including avenanthramides (AV-B) and phenolic acid (as trans-Ferulic Acid). Expression was modulated by significant inhibition of CREB, ERK and ATF transcription factors, which occurred when treated with any of the polyphenols. However, although we found small changes, contrary to some published studies, we found no significant inhibition of ACE activity via this mechanism, nor any significant increases in total NO, nitrite or nitrate. Therefore, we did not find conclusively that polyphenols reduce BP via the RAAS, however, we suggest that higher doses should be tested, as they may result in ACE inhibition.

In an acute randomised controlled crossover intervention trial (RCT) oat intake (90.2 g oats containing 50 mg phenolic acid) improved % FMD, however, while the improvements may have been medically relevant, they were not significant and were, therefore, inconclusive. Similarly, secondary outcomes including, notably, blood pressure and endothelium-independent vasodilation at early time points tended towards improvement; but the trials were not assessed

for power against secondary outcomes which, along with the lack of significance in the primary outcomes, prevents conclusions being drawn based on these results.

Similar outcomes which may signify lower stress on the vascular systems of the subjects, but were not statistically significant, were found from a chronic trial, where volunteers consumed different levels of oat based avenanthramides and phenolic acids. In particular, 24 hour ambulatory blood pressure and % FMD responses improved, as did night-time systolic blood pressure, which reduced by 5.1 mm Hg following a high phenolic oat intervention. High phenolic oats interventions also led to decreases in daytime and 24 h SBP by 0.15 and 1.16 mm Hg respectively and increased endothelial microvascular reactivity.

We conclude that there while there were indications of positive, medically relevant differences in vascular function, following both acute and chronic trials, none was statistically significant. The most marked improvements were seen in endothelium-independent blood flow at 2 h post consumption in the acute trial and lowered 24 h ambulatory BP in the chronic trial. The relatively short duration of the trials or likely too small, insufficiently powered sample sizes may have been responsible for the lack of conclusive statistical evidence.

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List of Abbreviations and Conventions

Abbreviation	Description
°C	Degrees Celsius
µL	Microlitre
ABPM	Ambulatory Blood Pressure Monitoring
ACE	Angiotensin Converting Enzyme
Ach	Acetylcholine
ADH	Antidiuretic Hormone
ADME	Absorption, Distribution, Metabolism and Excretion
AF	Average Fluorescence
Akt	Protein Kinase B
AMPK	Adenosine Monophosphate-activated Protein Kinase
Ang I	Angiotensin I
Ang II	Angiotensin II
ATF-1	Cyclic AMP-dependent Transcription Factor
AT1R	Angiotensin II Type I Receptor
AUC	Area Under Curve
Avn	Avenanthramides
Avn-c	Avenanthramide-C
AX	Arabinoxylan
BMI	Body-Mass Index
BP	Blood Pressure
CA	Ca^{2+}/calmodulin-dependent protein kinase II
CAD	Coronary Artery Diseases
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
cGMP	Cyclic Guanosine Monophosphate
CHD	Chronic Heart Disease
COX-2	Cyclooxygenase
CRE	cAMP-Responsive Element
CREB	cAMP Regulatory-Binding Protein
CREM	cAMP-Responsive Element Modulator
CRP	Plasma C-Reactive Protein
CVD	Cardiovascular Disease
D	Day
DBP	Diastolic Blood Pressure
DF	Dietary Fibre
ECL	Enhanced Chemiluminescence
EDRF	Endothelium-Derived Relaxing Factor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal-Related Kinase
ET-1	Endothelin-1
FA	Ferulic Acids

FAPGG	N-[3-(2-Furylacryloyl]-L-Phenylalanyl-Glycyl-Glycine)
FMD	Flow-Mediated Dilatation of the Brachial Artery
g	Gram
GAE	Gallic Acid Equivalents
GBD	Global Burden of Disease
GG	Glycylglycine
HDL	High-Density Lipoprotein
HDL-C	High-Density Lipid Cholesterol
Hg	Mercury
HR	Heart Rate
HRT	Hormone Replacement Therapy
HUVEC	Human Umbilical Veins
iAUC	Incremental Area Under Curve
IC_{50}	The Concentration of Inhibitor Required to Inhibit 50 % of the Renin Activity
IHD	Ischaemic Heart Disease
ISTD	Internal Standards
JG	Juxtaglomerular
JGA	Juxtaglomerular Apparatus
K	Potassium
kcal	Kilocalorie
kg	Kilogram
L	Litre
LDI	Laser Doppler Iontophoresis
LDL	Low-Density Lipoprotein
LDL-C	Low-Density Lipid Cholesterol
LOX-1	Lectin-Like Oxidised LDL Receptor-1
MAPK	Mitogen-Activated Protein Kinase
MCP	Monocyte Chemoattractant Protein
mg	Milligram
ml	Millilitre
mm	Millimetre
mmol	Millimole
MMPs	Metalloproteinase Enzymes
MRM	Multiple Reaction Monitoring
MTT	Methyl Tetrazolium
Na	Sodium
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ng	Nanogram
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NOx	Nitrite/nitrate
ox-LDL	Oxidised Low-Density Lipoprotein
P	Probability
PA	Phenolic Acid
pg	Picogram
PGI2	Prostacyclin
PGIS	Prostacyclin/Prostaglandin I 2 Synthase
Phenolic Oats	Intervention for consumption comprising

	Avenanthramides and Phenolic Acid (Ferulic Acid)
PKA	Protein kinase A
PRA	Plasma Renin Activity
PWA	Pulse Wave Analysis
RAAS	Renin–Angiotensin–Aldosterone System
RCT	Randomised control trial
ROS	Reactive Oxygen Species
RT	Room Temperature
SBP	Systolic Blood Pressure
SHR	Spontaneously Hypertensive Rats
SNP	Sodium Nitroprusside
TAG	Triglycerides
TC	Total Cholesterol
t-FA	Trans-Ferulic Acid
TP	Total Polyphenol
TNFα	Tumour Necrosis Factor-Alpha
TXA2S	Thromboxane A2 Synthase
WHO	World Health Organisation
WKR	Wistar-Kyoto Rats
y	Year

List of Publications and Presentations

Publications

1. Schär, M.Y., Corona, G., Soycan, G., Dine, C., Kristek, A., **Alsharif, S. N.**, Behrends, V., Lovegrove, A., Shewry, P. R. and Spencer, J. P., 2018. Excretion of Avenanthramides, Phenolic Acids and their Major Metabolites Following Intake of Oat Bran. *Molecular nutrition & food research*, *62*(2), p.1700499.

2. Dodd, G. F., Rees, A. J., Soycan, G., Alroqaiba, N. A. A., **Alsharif, S. N.**, Kristek, A., Currie, K., Spencer, J. P. E. Cerebrovascular disease: Possible protective effects of polyphenols and potential mechanisms of action (In preparation).

Poster presentations

1. (Poly)phenols inhibit renin activity and expression in mouse kidney cells: a potential mechanism underlying the blood pressure benefits of polyphenols?

   Sarah Alsharif, Gessica Serra, Manuel Schär, Gunter Kuhnle, Jeremy Spencer

   The 1st International Conference on Food Bioactives and Health. Norwich, UK from 13th to 15th September 2016.

2. Do oat phenolics improve blood pressure and vascular function in stage-1 and pre-hypertensive men through inhibition of the renin-angiotensin system?

   Sarah Alsharif, Manuel Schär, Angelika Kristek, Gulten Soycan, Hanna Petsch, Peter Shewry, Gunter Kuhnle, Jeremy Spencer

   8th International Conference on Polyphenols and Health. Québec City, Canada From October 3 to 6, 2017.

3. (Poly)phenols inhibit renin activities and expressions in mouse kidney cells: a potential mechanism underlying the blood pressure benefits of polyphenols?

   Sarah Alsharif, Gessica Serra, Manuel Schär, Gunter Kuhnle, Jeremy Spencer

   8th International Conference on Polyphenols and Health. Québec City, Canada From October 3 to 6, 2017.

Chapter: 1 Introduction

1.1 OVERVIEW

Cardiovascular disease (CVD) has a significant detrimental impact on human health, and is considered by the World Health Organisation to be the leading cause of mortality globally, (WHO, 2018), leading to 17.7 million or 31% of all deaths annually [8-11]. The most recent published Global Burden of Disease (GBD) figures for 1990 to 2015 show that CVD is prevalent in world-wide, and that, like the whole of Western Europe, the UK age-standardised death rate over that period was 91-220 per 1 x 10^5 of the population [8]. The figures also showed, however, a significant decline. Townsend *et al.* (2016), also based on the same WHO data, but in which information for 41 of the 52 European countries derives from 2010 or thereafter, reported that CVD accounted for 45% and 34% of all mortality in Europe and the UK respectively in 2015 [12]. Of world-wide mortality, an estimated 7.4 million deaths were due to coronary heart disease and 6.7 million were due to stroke; and over three-quarters of CVD deaths take place in low- and middle-income countries [13]. Furthermore, of the 17 million premature deaths (under the age of 70) due to non-communicable diseases in 2015, 82% are in low- and middle-income countries, and 37% are caused by CVD. In Europe, 3.9 million people die of CVDs each year, high systolic blood pressure (SBP) is the greatest medical risk factor, while diet was the most easily modifiable risk factor [13].

Cardiovascular diseases (CVD) are a major global health burden and create a substantial barrier to quality of life [8, 14]. A wide range of risk factors contribute to CVDs, including behavioural factors such as smoking, unhealthy diet, insufficient physical activity, and alcohol consumption, and physiological factors such as high BMI (body mass index), high total cholesterol, high blood pressure, and high fasting plasma glucose [13, 15, 16]. It is well established that one of the most important prognostic risk factors for cardiovascular disease risk is elevated blood pressure [17-20]. In particular, clinical data suggest that the maintenance of a healthy blood pressure is strongly associated with a reduced risk of CVDs [18, 21, 22]. Furthermore, the early detection and treatment of high blood pressure is thought to be an effective strategy for the prevention of early major cardiovascular events [23-27]. Notably, meta-analysis of existing studies indicate a decrease in ischaemic heart disease mortality risk by 30 % and stroke death by 40 % for a 10 mmHg reduction in systolic blood pressure (SBP) and/or 5 mmHg reduction in diastolic blood pressure (DBP) [28]. Indeed, the risk of developing CVD (relative risk [RR] 0·80, 95 % CI 0·77–

0·83), coronary heart disease (0·83, 0·78–0·88) or heart failure (0·72, 0·67–0·78) are all significantly reduced for every 10 mm Hg reduction in SBP [18].

People with CVD or who are at high risk of cardiovascular events from one or more risk factors, such as hypertension, diabetes and hyperlipidaemia, need early detection and management with both medical counselling and treatment [13, 15, 18, 22, 29, 30]. With respect to treatments, as well as pharmacological treatment, a number of clinical trials have suggested that hypertension may be modifiable through the improved diet, notably reducing high saturated fat and increasing the intake of plant foods [31-37]. A poor diet, rich in high-fat, processed foods and low in plant foods may impact directly on CVD risk via actions on a number of prognostic risk factors, such as blood pressure (BP), or indirectly by contributing to the development of obesity and/or diabetes which themselves lead to elevated BP [13, 38-41]. Polyphenols have been found to reduce CVD risk factors [42-52]; they are naturally occurring secondary metabolites found ubiquitously in plants. Their functions include: aiding growth and reproduction, providing defence against pathogens and protecting against environmental stressors. In addition, they contribute significantly to the sensory properties of fruits and vegetables and of products derived from them [53-55]. Polyphenol-rich foods decrease the incidence of coronary heart disease, inflammation and cancer [56-59].

It is well established among clinicians that early detection and control of high BP contributes to the prevention of major cardiovascular events [17, 24]. A London based study comparing electronic health records from 1997 to 2010 confirmed the importance of BP control, reporting that people with a systolic BP of 90-114 mm Hg presented the lowest risk of being diagnosed with cardiovascular diseases and that high SBP presents higher risks for intracerebral haemorrhage, angina, peripheral arterial disease and myocardial infarction in comparison to high DBP [20]. It has been asserted that a combination of medications directed towards reducing four risk factors associated with CVD (high BP, high low-density lipoprotein cholesterol, high serum homocysteine and platelets), may help to prevent 80 % of adults over the age of 55 developing the disease [60, 61]. However, the regular intake of a multi-drug treatment to influence these risk factors is in itself associated with several underlying complications, particularly with respect to the liver [62]. Consequently, people are increasingly recognising the potential benefits of diet as a substitute to, or a way of reducing the requirement for, complex drug treatments.

This chapter reviews the aetiology of CVD and the potential benefits that diet, in particular foods rich in plant polyphenols, can play in the prevention of these diseases.

1.2 CARDIOVASCULAR DISEASE

CVD usually involves blood vessels and/or the heart and encompasses coronary artery disease (CAD) such as myocardial infarction and angina, and heart failure, rheumatic heart disease, stroke, hypertensive heart disease, peripheral artery disease, cardiomyopathy, arrhythmia, valvular heart disease, congenital heart disease, aortic aneurysms, carditis, thromboembolic disease, and venous thrombosis, among others [13, 63, 64]. Below the age of 65 years, most deaths from CVD are considered preventable (Townsend *et al.*, 2016), amounting to 80%, including strokes, according to WHO (2018) [12, 65, 66]. Many types of CVD are considered to be preventable with appropriate lifestyle changes [15, 67]. Much research evidence points to the strong likelihood that cardiovascular health is dependent to a large extent on the normal function of the vasculature that supplies blood to the heart and other vital organs. It is thus of crucial importance to understand the pathogenesis of vascular conditions that lead to peripheral artery disease and strokes. Both are associated with endothelial dysfunction and atherosclerosis.

1.2.1 ENDOTHELIAL DYSFUNCTION AND ATHEROSCLEROSIS

The vascular endothelium is not just a physical barrier; it performs a range of functions including the release of a variety of agents to regulate vessel function. These include vasodilators (i.e. nitric oxide (NO), prostacyclins), vasoconstrictors (i.e. endothelin-1, prostaglandins), endothelial and smooth muscle cell growth regulators (i.e. fibroblast growth factor, endothelin, transforming growth factor, platelet-derived growth factor, heparin and heparan sulphate) and factors influencing platelet and leukocyte interactions (i.e. ICAM, VCAM and integrins) [68-75]. Loss of homeostasis of the vasculature (endothelial dysfunction) may arise from any adverse vascular events, including: physiological changes including reduced bioavailability of vasodilators derived from the endothelium, especially NO; increased bioavailability of contracting factors derived from the endothelium; changes in endothelium permeability; amplified expression of adhesion molecules on the surface of endothelial cells; reduced antithrombotic factor secretion; increased pro-coagulation factor production; and reduced endothelial antioxidant and anti-inflammatory capacity [76, 77]. Endothelial dysfunction leads to atherosclerotic processes in the blood vessel walls [78-81]. Atherosclerosis, the development of atheroma in arteries, also known as arteriosclerotic vascular disease, is a condition in which deposits of fatty material and plaques build up inside arteries [82, 83]. Plaques are composed of cholesterol, fat, calcium and other substances found circulating in the blood [84]. As the plaque increases in size, it becomes less pliable and can severely narrow an artery, thus limiting the flow of oxygen-rich blood to vital organs [83, 85]. In more advanced plaques, intracellular micro-calcifications also form within vascular smooth muscle cells of the surrounding muscular layer, specifically in the muscle cells

adjacent to the atheroma and as cells die, this leads to extracellular calcium deposits between the muscular wall and outer portion of the atheromatous plaques [82, 86, 87]. Although the disease process tends to be slowly progressive over time in medium-sized arteries, it usually remains asymptomatic until an atheroma ruptures, causing cardiac abnormalities such as acute myocardial infarction, unstable angina and ultimately sudden cardiac death [77, 82, 83, 87].

One of the processes thought to initiate the progression of atherosclerosis (Figure 1.1) is inflammation, which may lead to the oxidation of low-density lipoproteins (LDL) and their ingestion by monocytes [77, 88, 89]. These cholesterol-laden monocytes, promoted by recruiting factors such as VCAM-1, enter the artery wall, becoming "foam cells" which eventually rupture, depositing a greater amount of oxidised cholesterol into the artery wall. This triggers more proliferation of white blood cells and further inflammation, thus perpetuating the cycle [83, 89].

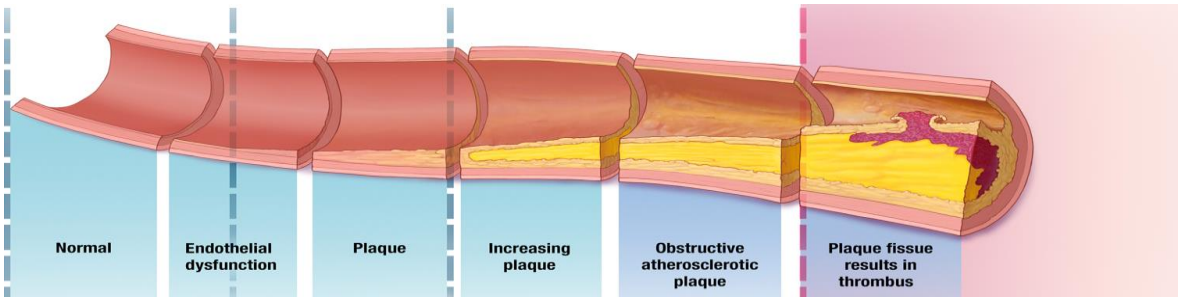


Figure 1.1 Diagrammatic representation of the typical progression of atherosclerosis in an artery
Reproduced from Widmer *et al.*, 2016 [90].

Contributing risk factors for the progression of atherosclerosis include adherence to a 'western diet', characterised by the consumption of processed food and high-fat content foodstuffs [41]. A sedentary lifestyle also contributes, as do diabetes, obesity, smoking and excessive alcohol consumption [38, 77, 86, 91, 92] all of which lead to high BP, and high levels of circulating cholesterol and reactive oxygen species (ROS) [93, 94]. Atherosclerosis can be prevented and improved in a number of ways, notably by increasing the level of exercise, improving diet, the cessation of smoking and by limiting alcohol intake, it is also important to take appropriate measures to reduce the levels of associated health-related risks such as high blood lipid levels, diabetes and/or high BP [18, 25, 95-97].

1.2.2 RISK FACTORS ASSOCIATED WITH ENDOTHELIAL DYSFUNCTION

It is necessary to understand the mechanisms of action of cholesterol, BP, Renin-Angiotensin-Aldosterone system (RAAS), nitric oxide (NO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, on the endothelium, all of which are linked to CVD risk factors.

1.2.2.1 CHOLESTEROL

Cholesterol, an isoprenoid lipid, is involved in two major biological processes. It provides structural support to cell membranes and myelin and it also acts as precursor from which oxysterols, steroid hormones, vitamin D_3 and bile acids are derived [98]. Cholesterol is synthesised *de novo* by liver and intestinal epithelial cells or is derived from dietary lipids [98, 99]. The amount of cholesterol absorbed from the diet is approximately 400 mg/day and from the bile it is approximately 1 g/day [99]. Fifty percent of dietary cholesterol is absorbed and the rest excreted. Both metabolic as well as genetic factors regulate cholesterol absorption in humans [99, 100].

Synthesis of cholesterol takes place via the isoprenoid biosynthetic pathway [101]. In the initial reactions of the pathway, HMG-CoA is formed from acetyl-CoA in the cytosol of liver cells. After some enzymatic reactions, HMG-CoA is converted into isopentenyl pyrophosphate, the basic isoprenoid carbon building block of cholesterol. The reaction is catalysed by HMG-CoA reductase [102]. Isopentenyl pyrophosphate is further reduced to squalene (composed of 6 isoprene units). The final stage of cholesterol biosynthesis, where squalene cyclisation takes place to form C_{30} lanosterol, requires molecular oxygen. Generation of C_{27} cholesterol from lanosterol requires at least 8 separate enzyme reactions [101]. Some enzymes involved in the cholesterol biosynthesis such as HMG-CoA reductase are present in the endoplasmic reticulum, whereas others are localised in the cytosol [99].

Serum lipoproteins transport cholesterol intra- as well as extra-vascularly. Lipoproteins generated by the liver and intestine transport cholesterol between the intestine and the liver, and between the liver and peripheral cells [103]. The general structure of mature lipoproteins is in the form of emulsion particles which have a core of neutral lipids, such as triacylglycerol (TAG), cholesteryl ester (CE) and cholesterol; stability of the core is maintained by outer layer of phospholipids, cholesterol and apolipoproteins. Apolipoprotein B100 (ApoB100) is the main protein component of LDL. LDL receptors on the cells recognise and internalise ApoB100-containing lipoproteins incorporating TAG and cholesterol. Hence ApoB100-containing LDL particles play an important role in atherogenesis. High-density lipoproteins (HDL) are key mediators in reverse cholesterol transport (RCT). In RCT, extrahepatic cholesterol is transported to the liver and excreted from the

body in faeces, therefore, HDL particles help prevent atherosclerosis. ApoA-I, a constituent protein of human HDL, has important roles in processes including discoidal HDL particle generation, remodelling of HDL by lecithin cholesterol acyltransferase (LCAT) and interaction with scavenger receptor class B type 1 (SR-BI), all of which contribute to cholesterol delivery in RCT [104]. Cholesterol cannot be catabolised by cells in oxidative processes. Hence disposal of excess cholesterol involves transportation to the liver and conversion into excretable bile acids that are eliminated from the intestine [98, 104, 105]. Cholesterol homeostasis is achieved by various feedback mechanisms. Increased cholesterol levels modulate HMG-CoA reductase and suppress cholesterol biosynthesis. High cholesterol levels also affect expression of LDL receptor and lower cholesterol uptake. Cholesterol levels modulate activity of cholesterol 7 alpha-hydroxylase which in turn modulates cholesterol catabolism [105].

High levels of circulating blood cholesterol are significantly correlated with people who developed CVD, mainly due to the contribution of LDL particles to atherosclerosis [85]. Atherosclerosis is often associated with elevated levels of oxidised LDL molecules, particularly small dense LDLs [93, 94, 106]. An abnormal lipid profile is characterised by high LDL-C and triglycerides and low HDL-C concentrations in plasma [106]. HDL molecules are believed to be an important mechanism for removal of cholesterol from atheromas, and elevated levels of HDL are associated with reduced rates of atheroma progression, and, in some cases, regression [86]. The long-term impacts of serum cholesterol levels on mortality associated with coronary heart disease (CHD) and CVD were studied in healthy younger males; CHD increased 2.15 to 3.63 times in men with greater than or equal to 240 mg/dL cholesterol compared to standard (< 200 mg/dL); levels; corresponding CVD mortality risk increased 2.10 to 2.87 times [107]. A life expectancy gain of 3.8 to 8.7 years was estimated in males with normal cholesterol levels. HDL cholesterol (HDL-C) levels were inversely associated with mortality in the Framingham Heart Study and follow-up studies: the relative risk of death was 3.6 for CVD and 4.1 for CHD in men when HDL-C levels were > 54 mg/dL compared to < 35 mg/dL. In women, relative risk of death was 1.6 for CVD and 3.1 for CHD when HDL-C levels > 69 mg/dL compared to < 45 mg/dL) [108]. These figures represent a two percent reduction in atherosclerosis risk for every 1% reduction in LDL-C level [109].

The Multiple Risk Factor Intervention Trial (MRFIT) showed that cholesterol levels in diet are significantly and directly linked to diastolic blood pressure (DBP) as well as (SBP), while the Western Electric Study showed that average annual increases in BP were directly and significantly associated with dietary cholesterol levels [100]. The INTERMAP study showed that cholesterol intake levels were in direct proportion to SBP, but not correlated with DBP. SBP differences of 0.9 mm Hg between baseline and 2 standard deviation higher cholesterol consumption

(131.0 mg/1,000 kcal) were estimated across all participants, with, however, differences of 1.1 mm Hg in participants who did not have hypertension. Cholesterol levels in food varies between populations and the association of dietary cholesterol and BP is not yet clearly established across different communities with diverse diets, ethnicities and genders [100]. Of note, in relation to this study, dietary polyphenols have been shown to reduce TAG, LDL-C and total cholesterol [48, 110] and flavonoids have been inversely correlated with LDL-C and total cholesterol [111].

1.2.2.2 BP

BP pertains to the pressure within arteries, as the result of blood forced into circulation throughout the body. SBP is the maximum pressure during ventricular contraction whereas DBP is the minimum pressure preceding the subsequent contraction [112], they are generally expressed as SBP/DBP mm Hg. A major risk factor for CVD is long-term high BP or hypertension [113, 114], which is classified as either primary, resulting from non-specific genetics and lifestyle factors, or secondary, resulting from particular causes, such as the development of atherosclerosis, chronic kidney disease or endocrine disorders [115]. High BP (hypertension) was previously defined as persistent resting BPs of 140/90 mm Hg or above [115, 116].

However, after extensive consultation, these guidelines have been revised [22] and four new BP categories have been introduced.

1. Normal BP: SBP < 120 and DBP < 80 mm Hg
2. Elevated BP: SBP 120-129 and DBP < 80 mm Hg
3. Stage 1 Hypertension: SBP 130-139 or DBP 80-89 mm Hg
4. Stage 2 Hypertension: SBP ≥ 140 or DBP ≥ 90 mm Hg

The new US guidelines, which have lower thresholds than NICE recommendations (2016), mean that there will be a substantially higher diagnosis of high BP in the USA, 46% of adults compared to 32% in the UK. However, for most US adults meeting the new definition of hypertension, non-pharmacological treatment is recommended. Since most people between 130-139 mm Hg SBP or 80-89 mm Hg DBP will not require medical treatment; there will only be a small increase in the percentage of adults for whom antihypertensive medication is recommended, albeit also in conjunction with lifestyle modifications [22].

Hypertension involves the activation of the sympathetic nervous system and the RAAS [117]. Almost 30% of UK adults have hypertension, and BP patterns inevitably change with age [118]. Hypertension related diseases cost the NHS approximately £2 billion per year, with extended raised BP being linked to a higher risk of renal failure, CVD and cardiovascular related mortality.

Longitudinal data from the Framingham Heart Study showed that SBP/DBP at 130–139/85–89 mm Hg, more than doubles the risk of CVD, when compared to a normal BP range [22, 119]. By reducing BP by just 10 mm Hg systolic or 5 mm Hg diastolic, the risk of stroke occurrence is reduced by 41%, and CHD by 22 % [21]. Beneficial dietary interventions include reducing sodium intake [120], a Mediterranean diet [121], and the inclusion of dietary proteins from plants [122], low fat foods [123], fish oils [124], monounsaturated fats [125], potassium derived from fruit and vegetables [126] and polyphenols [127].

1.2.2.3 RENIN–ANGIOTENSIN–ALDOSTERONE SYSTEM

The RAAS is a hormonal system that regulates the concentration of plasma sodium, and is critical in arterial BP homeostasis (Figure 1.2) [128-131]. The RAAS process functions in a cascade, leading to the generation of angiotensin II (Ang II), which is a major vascular effector [129]. Therefore, the RAAS is important for the study of hypertension and associated CVD [128, 132]. The RAAS precursor molecule is Angiotensinogen, a protein produced mainly by the liver, angiotensin I (Ang I) is formed by cleavage of the first 10 amino acid peptides from Angiotensinogen by the aspartyl protease renin [133, 134].

Renin is secreted from juxtaglomerular cells in the kidney (Figure 1.3), which sense changes in renal perfusion pressure through signals produced by stretch receptors in the vascular walls, it is the rate-limiting step in the activation of the RAAS [128, 133, 134]. The juxtaglomerular cells also release renin in response to signals from the *macula densa* located in the distal nephron, as a result of changes in blood flow [135]. The juxtaglomerular apparatus in the renal cortex represents a major structural component of the RAAS and is one of the most important regulatory sites of renal salt/water conservation and BP maintenance [136]. Baroreceptors in the kidneys are able to detect reductions in BP, sodium ion (Na^+) levels or blood volume leading to an increase in sympathetic tone and the release of renin [137]. A schematic illustration of the juxtaglomerular apparatus (JGA) is shown in Figure 1.3.

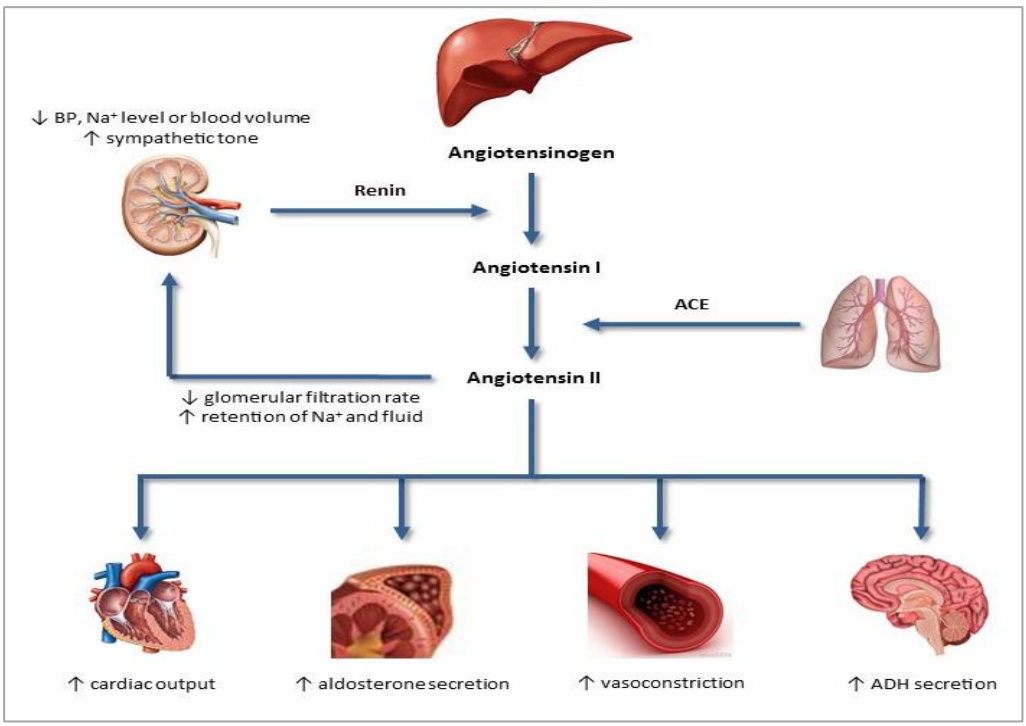


Figure 1.2 Diagram depicting the renin-angiotensin-aldosterone system (RAAS).

Baroreceptors in the kidneys are able to detect reductions in blood pressure (BP), sodium (Na+) levels or blood volume leading to an increase in sympathetic tone and the release of renin. Plasma renin converts angiotensinogen, a precursor protein made in the liver, into angiotensin I. This is then converted by angiotensin-converting enzyme (ACE), particularly found in the lungs, into angiotensin II (Ang II), a potent vasoconstrictive peptide. Ang II is able to exert effects in various organs and tissues throughout the body in order to increase blood pressure. In the kidneys, Ang II decreases the glomerular filtration rate, whilst in the adrenal gland, secretion of aldosterone is stimulated in order to retain Na+ and fluids. Ang II also increases cardiac output, vasoconstriction in blood vessels and stimulates the hypothalamus to release antidiuretic hormone (ADH) from the posterior pituitary gland, which allows greater reabsorption of water in the kidneys.

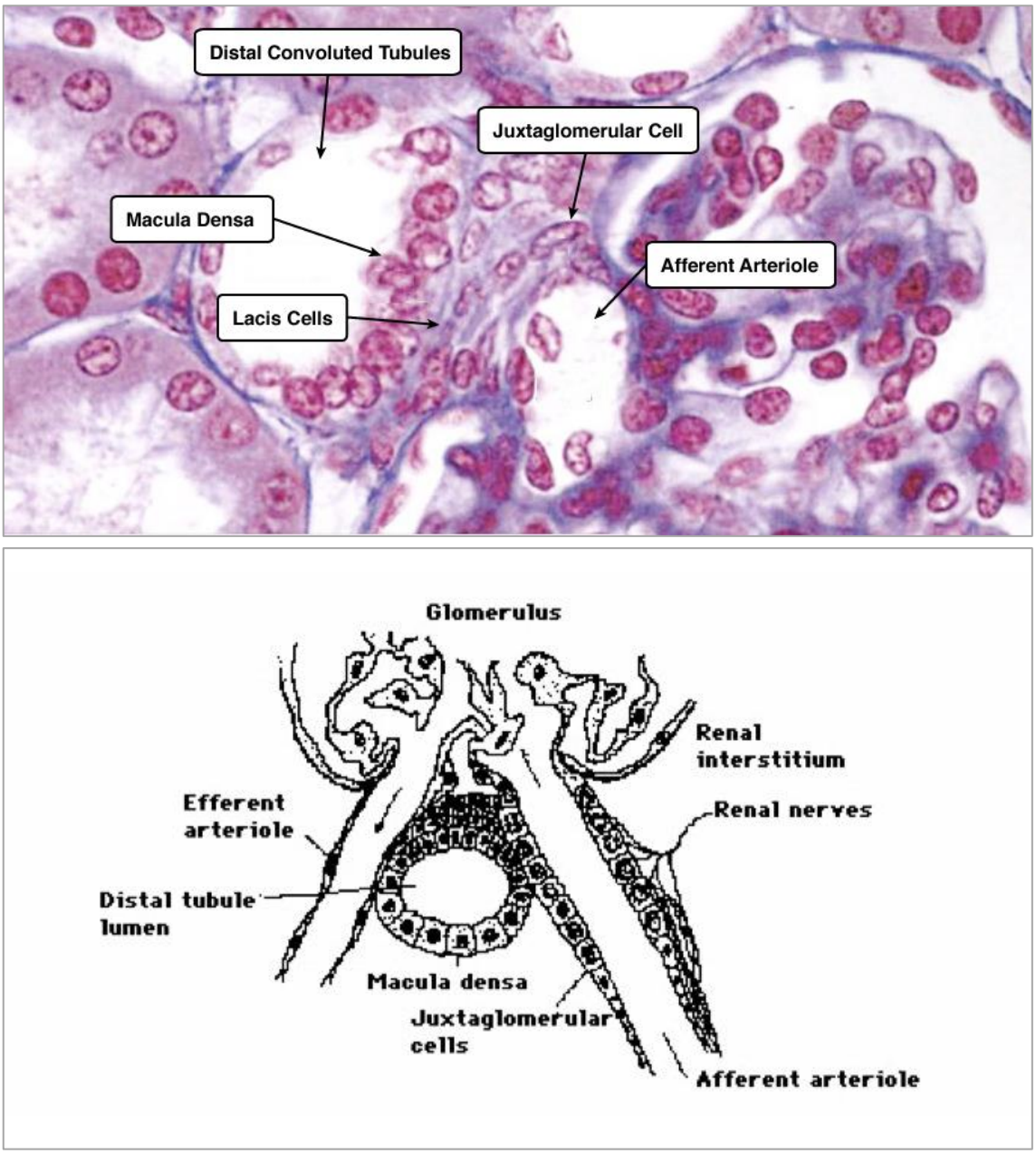


Figure 1.3 Microscopic Structure of the Juxtaglomerular Apparatus (JGA)

Upper image: high-power photomicrograph of the histology of the human renal glomerulus, stained with haematoxylin & eosin (H&E). Juxtaglomerular cells can be identified by their location and their histological appearance – they are large cells with pale, enlarged nuclei. (Adapted from Yale.edu teaching website: http://medcell.med.yale.edu/histology/urinary_system_lab/juxtaglomerular_...apparatus.php [Accessed – 1/6/2016].

Lower image: diagrammatic representation of the location of the juxtaglomerular cells in the wall of the afferent renal arteriole, which allows secretion of renin into the blood and renal lymph. Stretch receptors in the afferent arteriole and sympathetic nerve ends in the region of the JGA cells; these, and the renal tubular fluid reaching the *macula densa*, orchestrate the regulation of renin secretion. (Adapted from Davis, 1973).

Angiotensin converting enzyme (ACE), mainly found in the lungs, cleaves Ang I to a smaller, highly active, 8 amino acid peptide Ang II, which is a potent vasoconstrictor, causing the narrowing of blood vessels and a rise in BP, as well as stimulating the secretion of aldosterone from the adrenal cortex [134, 136, 138, 139]. Ang II has actions on various organs and tissues throughout the body, including the kidneys, where Ang II decreases the glomerular filtration rate [131]. It also increases cardiac output, vasoconstriction and stimulates the hypothalamus to release antidiuretic hormone (ADH), also termed arginine vasopressin, from the posterior pituitary gland, which leads to greater reabsorption of water by the kidneys [140]. ADH acts on the renal collecting ducts by stimulating Vasopressin V2 receptors, which increases water permeability through a Cyclic Adenosine Monophosphate (cAMP)-dependent mechanism. As a consequence, volume of blood is increased together with cardiac output, in addition, aldosterone acts on the renal tubules, resulting in an increased reabsorption of Na^+ and H_2O into the blood, while maintaining electrolyte balance by excreting K^+ [141]. The overall result is an increase in extracellular fluid volume within the body, and hence increased BP [140, 142, 143]. Thus, any abnormality or unwarranted increase in RAAS function results in hypertensive vasoconstriction and reabsorption of sodium ions.

ACE inhibitors and angiotensin II receptor blockers are effective at reducing BP in hypertensive individuals, even where there is no systemic RAAS activation [144-146]. Ang II localised within the kidneys is regulated independently from levels in the systemic RAAS and concentrations within the kidneys are higher than in blood plasma and can reach even greater levels in experimental hypertension models [147]. Systemic and kidney Ang I receptors contribute separately to baseline BP [148], furthermore, the presence of renal Ang I receptors determines long-term sensitivity to Ang II-induced hypertension [149]. Absence of renal ACE has been demonstrated to provide protection against hypertension, irrespective of plasma Ang II levels. In hypertension induced by Ang II, the shifting of the renal pressure–natriuresis relationship, whereby higher renal perfusion pressure leads to lower sodium reabsorption and higher sodium excretion, is largely dependent on renal ACE, since Ang II is produced locally [150].

1.2.2.4 NITRIC OXIDE

Vascular endothelial cells secrete numerous chemicals including NO that affect the regulation of vascular homeostasis. The role of NO is pivotal in the regulation of vascular homeostasis [6], and is definable as the molecule's continuous basal low level release into the endothelium through relaxation of vascular smooth muscle cells mediated by cyclic guanosine monophosphate (cGMP),

leading to vasorelaxation [151-154]. NO is, therefore, important as a BP and blood flow regulator [155].

NO, previously called endothelium-derived relaxing factor (EDRF) until its identity was unequivocally established in 1987 by Palmer [156, 157], is one such crucial chemical, and was initially described as a vasodilator derived from the endothelium [158]. This proposal was based on the observation that superoxide dismutase (SOD), which removes O_2^-, protected EDRF from rapid inactivation and that haemoglobin selectively inhibited EDRF [159], as well as on a study of the transient relaxations of endothelium-denuded rings of rabbit aorta to 'acidified' inorganic nitrite (NO_2^-) solutions. Despite the technical difficulties related to the low solubility of NO in water, its reactivity with oxygen and its instability, it was observed that EDRF and NO resembled one another in several pharmacological tests, including their half-lives, stabilisation by SOD and inhibition by haemoglobin [157].

NO synthesis from the amino acid L-arginine is through the L-arginine endothelial nitric oxide synthase (NOS) pathway by endothelial nitric oxide synthase (eNOS) [152, 160-163]. NO can also be generated *in vivo* by the nitrate-nitrite-nitric oxide pathway [164-166]. NO reacts with the haem group on an enzyme called guanylyl cyclase. On activation, this enzyme catalyses the production of cGMP and finally cGMP activates protein kinase, an enzyme that phosphorylates other proteins to alter their activity, leading to relaxation [167-169]. High concentrations of NO also activate K-type Ca channels in the smooth muscle membrane, which cause hyperpolarisation and, therefore, vasodilation. An increase in cGMP in vascular smooth muscle cells derived from an influx of NO leads to dilation of the blood vessels [170]. NO is a free radical gas and its half-life *in vivo* is a few seconds [42].

Endothelial cells perform regulatory functions including regulation of vascular tone (Figure 1.4) and structure, and have anticoagulant, antiplatelet, and fibrinolytic properties [86]. NO, ROS and bradykinin, are vasodilatory molecules [171]. ROS prevent platelet aggregation, and together with bradykinin, promote the release of NO [171]. NO moderates many of the responses of the vasculature (Figure 1.5) and can overturn the effects of vasoconstrictors originating from the endothelium, as well as inhibiting oxidation of LDL leading to lowering of CVD [172]. Endothelial cells produce several vasoactive factors affected by NO, among them prostacyclin (PGI2) [70]. However, they also produce substances which act as vasoconstrictors, such as endothelin and Ang II (which acts as a pro-oxidant, stimulating the production of endothelin). Both chemicals encourage the proliferation of smooth muscle cells [173].

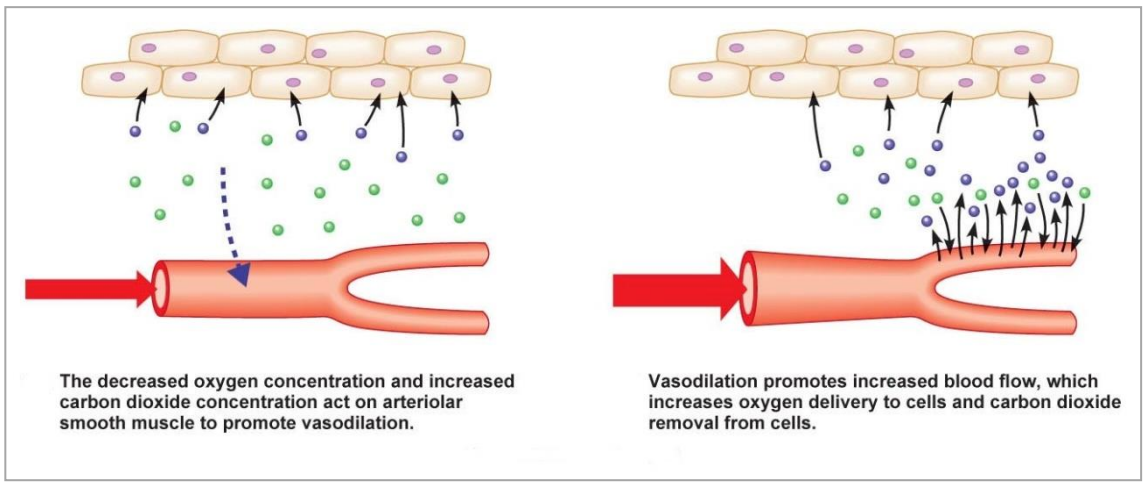


Figure 1.4 Vasodilation and its role in increasing blood flow and reducing blood pressure.

Blue and green dots represent carbon dioxide and oxygen concentrations, which act on arteriolar smooth muscle cells. Black and blue arrows show O_2 and CO_2 exchange. (After Stanfield *et al.*, 2011).

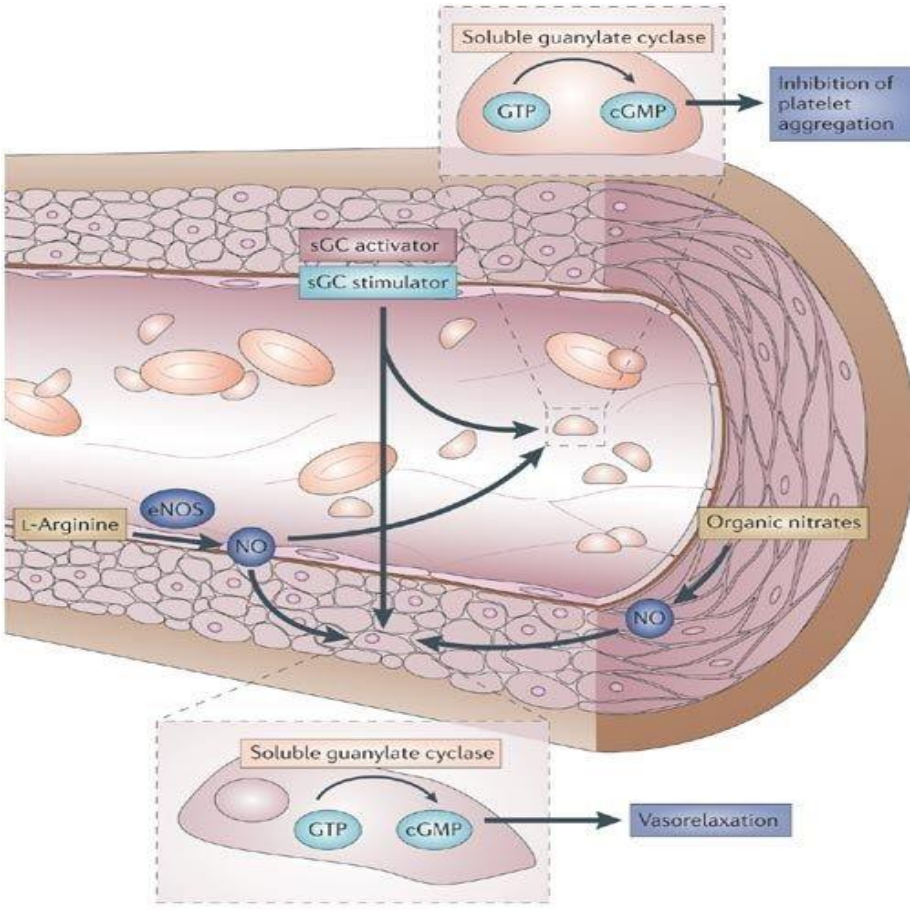


Figure 1.5 Nitric Oxide signaling in the vascular endothelium.

cGMP - cyclic guanosine monophosphate; eNOS - Endothelial Nitric Oxide Synthase; GTP - guanosine triphosphate; NO – Nitric Oxide; sGC - soluble guanylate cyclase. Reproduced from Evgenov *et al.*, 2006 [4].

The breakdown of optimal NO homeostasis, i.e. steady-state NO levels, is one of the most critical features underlying endothelium dysfunction [174] and may arise from dysfunction in eNOS leading to reduction in NO bioavailability, or in the availability of its substrate (i.e. L-arginine), its cofactors (i.e. tetrahydrobiopterin, flavin adenine dinucleotide, flavin mononucleotide and NADPH) or eNOS inhibitors (i.e. L-NAME) [70, 74, 75].

1.2.2.5 NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE OXIDASE

The membrane bound complex NADPH oxidase comprises a GTPase and different phox subunits. There are a number of ways NADPH oxidase can be expressed in the cardiovascular system and it is possible to activate subunits of the enzyme in CVD [175]. Moderate NADPH oxidase activity is an important part of angiogenesis and immunity, but an excess results in excess ROS causing endothelial dysfunction [176-178]. NADPH's primary function is superoxide and/or hydrogen peroxide (H_2O_2) generation. NADPH oxidase catalyses the reduction of O_2 to generate a superoxide anion (O_2^-) regarded as a major source of ROS. The interaction of O_2^- with other molecules to produce secondary ROS may be direct or may be catalysed by an enzyme; in either case the consequence of the reduction of O_2^- is that H_2O_2 is formed and then converted to hydroxyl (HO). An alternative O_2^- reaction is with NO, leading to the formation of peroxynitrite ($ONOO^-$), and then to the uncoupling of eNOS followed by reduced NO production, superoxide generation and increased oxidative stress [179]. Figure 1.6 illustrates the role of NADPH oxidase activity and NO bioavailability in the rise in oxidative stress which is known to be a factor in CVD.

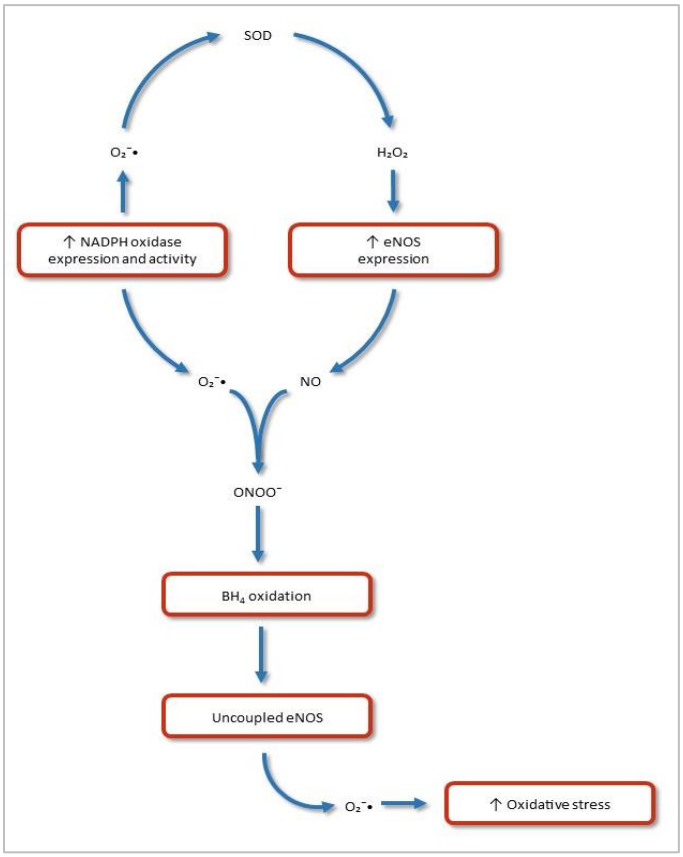


Figure 1.6 The role of NADPH oxidase activity and NO bioavailability in increasing oxidative stress.
NADPH oxidase, the main generator of superoxide (O_2^-), and eNOS expression are major factors upregulated in the pathophysiology of vascular disease. Hydrogen peroxide (H_2O_2) is produced with the dismutation of O_2^-, mediated by superoxide dismutase (SOD) and can lead to increased eNOS expression. O_2^- generated through NADPH oxidase activity and NO produced through increased eNOS activity react to form $ONOO^-$. $ONOO^-$ oxidises tetrahydrobiopterin (BH4), an essential cofactor of eNOS, causing uncoupling of eNOS. As a result, functional NO-producing eNOS is converted to a dysfunctional O_2^- generating enzyme which increases oxidative stress in the vascular system.
Adapted from Förstermann and Sessa, 2011 [6].

1.3 POLYPHENOLS

Polyphenols are secondary metabolites used by plants in defence against pathogens and ultraviolet radiation [57, 180]. There are over 8,000 structural variants of polyphenols, which are classified by biological function, origins, and chemical structure into four broad subgroups: flavonoids, lignins, phenolic acids and stilbenes, which are then, in some cases, further subdivided. Of plant polyphenols, flavonoids are the most abundant and widely distributed, with fruits such as apples, berries and grapes, and olives, dark chocolate, red wine and tea constituting the main human dietary sources [55, 181-183].

1.3.1 STRUCTURE

Polyphenols, also referred to as polyhydroxyphenols, are a specific structural group of naturally occurring organic chemicals comprised of multiple phenol units. Polyphenols are mostly natural macromolecular compounds; however, semi- and synthetic-polyphenols are now common [184]. One important subgroup of polyphenols, phenolic acids, is further classified into hydroxybenzoic acids and hydroxycinnamic acids, which include gallic, p-coumaric, caffeic, ferulic and sinapinic acids [185]. Flavonoids, another important subgroup, include anthoxanthins (flavones and flavonols), flavanones, flavanonols, flavans, anthocyanidins and isoflavonoids (Figure 1.7) [2, 186, 187]. The resveratrol in the stilbenoid subgroup is under active scientific investigation for its anti-carcinogenic properties [188].

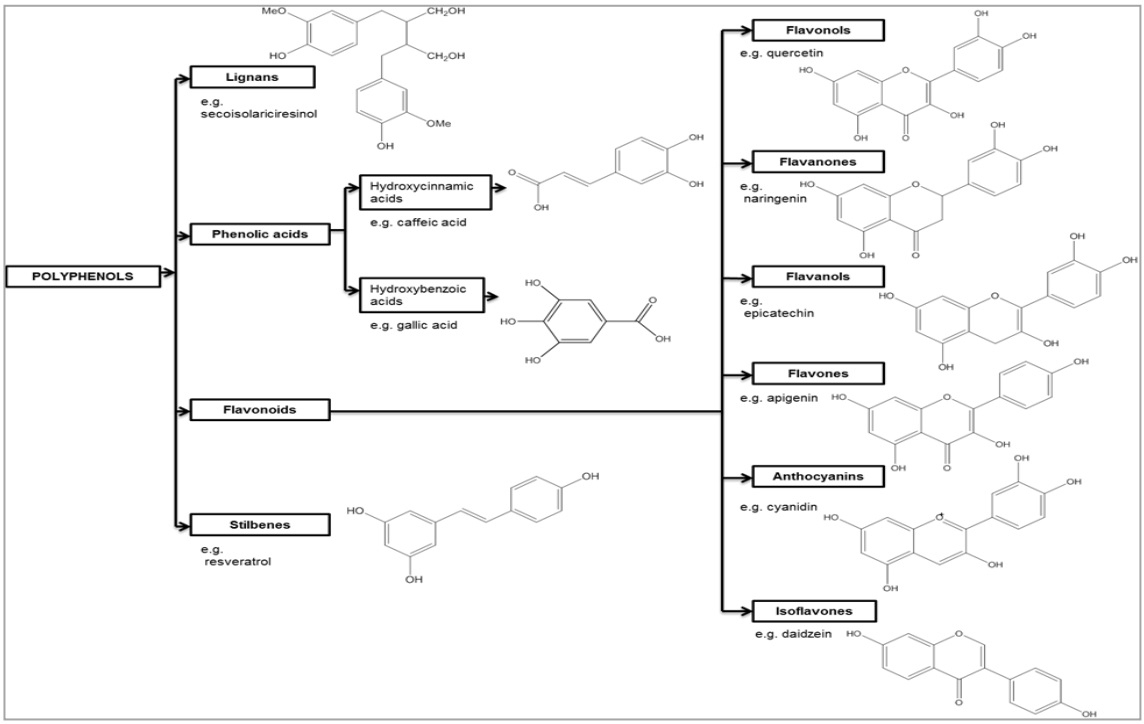


Figure 1.7 Classification of Polyphenols

Showing the four main groups of polyphenols and their chemical structures, as well as the subgroups of phenolic acids and flavonoids. (Adapted from Spencer *et al.*, 2008) [2].

1.3.2 DIETARY SOURCES

Polyphenols are abundantly available in normal, routine dietary sources including fruits and beverages such as coffee, tea and red wine [180, 183]. Some types of polyphenols, such as quercetin, are available in all plant sources, for example: fruits, vegetables (including legumes)

and cereals, as well as derived products such as tea. Other polyphenols, such as flavanones and isoflavones, are specific to certain citrus fruits, soya, and apples [189-192].

The consumption of polyphenols is dependent on diet and so varies between individuals and populations. An extensive European Prospective Investigation into Cancer and Nutrition (EPIC) study of dietary polyphenol intake in the European diet of more than 36,000 adults was carried out [193]. The mean total intake of polyphenols in a general population was highest in Aarhus (Denmark) at 1,786 mg/day in men and 1,626 mg/day in women and the lowest in Greece at 744 mg/day in men and 584 mg/day in women. The highest intake of total polyphenols overall was observed in the UK health-conscious group of subjects (1521 mg/day), followed by non-Mediterranean then Mediterranean countries. The biggest proportion of polyphenols consumed was phenolic acids (52.5 - 56.8%), except in men from Mediterranean countries and in the UK health-conscious group where they it was flavonoids (49 - 61.7 %). Coffee, tea, fruits and wine were the most important food sources of total polyphenols. A total of 437 different individual polyphenols were consumed, including consumed at a level of ≥ 1 mg/day. The most abundant ones were 5-caffeoylquinic acid, proanthocyanidin polymers, and 4-caffeoylquinic acid [193].

World-wide studies on dietary intake of polyphenols and total polyphenol content, and its variation between genders, have shown high levels of heterogeneity between countries; especially among flavonoids and phenolic acids [191]. Within Europe, apples, strawberries and potatoes are the main fruit and vegetable sources of polyphenols e.g. in the French diet [189, 193] while fruits, along with coffee, are the primary dietary source of polyphenols in the Spanish population, additional consumption in olives and olive oil differentiates this and other Mediterranean populations from others [194]. Of note, in this respect, the traditionally polyphenol-rich diet in Greece, resulting from vegetables (particularly wild plants), fruits, nuts, cereals, and moderate amounts of wine, also includes olives and olive oil [192]. In Portugal, the total polyphenol intake from fresh fruits has been estimated to be 783.9 ± 31.7 mg/day of gallic acid equivalents (GAE), of which 14% are from berries, particularly strawberries [190]. Studies of northern European diets, such as the Finnish, have shown that berries, coffee and cereals are the leading polyphenol sources [195]. The average intake of polyphenols in Poland has been estimated as 1,756.5 ± 695.8 mg/day, with flavonoids (897 mg/day) and phenolic acids (800 mg/day) the major polyphenols consumed [196]. Flavanols and hydroxycinnamic acids are the primary dietary polyphenols in the United Kingdom [193, 197]. Outside of Europe, there is evidence from the United States, that the average dietary polyphenol intake is about 1,000 mg/day, and from Brazil, where it is 1,198.6 mg/day [198]. In Japan it is 1,492 ± 665 mg/day [199] and in Korea, 318.0 mg/day, mostly from flavonoids [200].

1.3.3 ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION (ADME)

It has been shown that dietary polyphenols beneficially affect a range of chronic diseases, including CVD, but these compounds' biological properties *in vivo* will depend on their degree of biotransformation and conjugation as they are absorbed from the gastrointestinal (GI) tract, then in the liver and finally in cells [201, 202]. Understanding polyphenols' bioavailability and bioaccessibility is a key prerequisite for determining which phenolic metabolites may mediate health benefits [203]. Natural polyphenols are extensively metabolised following oral ingestion. Dietary polyphenols in the upper GI tract act as substrates for several enzymes and are extensively metabolised by glucosidase enzymes, phase I enzymes (hydrolysing and oxidising), such as cytochrome P450, and phase II enzymes (conjugating and detoxifying) which are found in the small intestine and liver [44, 204]. Further transformation in the colon has been reported, with enzymes in the gut microflora breaking flavonoids down to simple phenolic acids [205, 206]. Colonic microbiota are responsible for bio-converting bioactive compounds into metabolites of lower molecular weight which, on absorption, are likely to be the cause of the health benefits resulting from food rich in polyphenols [207, 208]. Metabolites that have entered the circulation are available for transport throughout the body and can, therefore, act on a variety of tissues including the endothelium.

a) Upper GI tract

Polyphenol structure may be modified at a number of points in the GI tract as the following examples show. In the upper GI tract, saliva causes degalloylation of flavanol gallate esters including epigallocatechin gallate, but has little effect on the stability of green tea catechins [209, 210]. The quercetin rutinoside, rutin, is hydrolysed by cell-free extracts of human salivary cultures [211] and by *streptococci* isolated from the mouths of normal individuals, but quercetin-3-rhamnoside (quercitrin) is not susceptible to hydrolysis and this suggests that only rutin-glycosidase-elaborating organisms occur in saliva [212, 213]. An interaction has been shown between flavanols and procyanidins with salivary proteins and this indicates that the affinity of (+)-catechin for proline-rich proteins is higher than that of than (-)-epicatechin and that C(4)-C(8) linked procyanidin dimers bind more strongly than their C(4)-C(6) counterparts [214]. This polyphenol-protein binding with salivary proteins of high molecular weight, bacterial cells and mucous materials may explain the decrease in quercetin mutagenicity seen after incubation with saliva [215]. Many factors influence the extent and rate of the small intestine's absorption of ingested compounds [216] including physiochemical factors such as molecular size, lipophilicity, solubility, pKa, and biological factors such as gastric and intestinal transit time, lumen pH, membrane permeability and first pass metabolism [217]. For some polyphenols, significant non-

enzymatic hydrolysis may occur at low gastric pH. Aglycone secoiridoids, for example, which occur in extra virgin olive oil, are subject to time-dependent hydrolysis in the acidic gastric environment which increases the longer they stay in the GI tract [218]. However, under normal pH conditions (pH 2.0) and normal physiological time frames (up to four hours) some remain intact and enter the small intestine without having been hydrolysed [218]. Procyanidin oligomers ranging from a dimer to decamer (isolated from Cocoa, *Theobroma cacao*), have shown instability under low pH similar to that present in the stomach's gastric juice [219]. Secoiridoid absorption from olive oil, as with flavanols and procyanidins after ingestion of chocolate or cocoa, are likely to be affected by earlier events in the gastric lumen within the residence time.

Glycosides which are polyphenol derivatives are relatively polar molecules and unlikely to diffuse passively across the membranes of the small intestinal brush border. It has, however, been suggested in a number of studies that β-glucosidases affect flavonoid glycosides before they are absorbed in the jejunum and ileum and removal of the glycosidic moiety is generally believed necessary before the flavonoid can be absorbed [220-226]. Most polyphenol glycosides, and sometimes aglycones, in foods derived from plants, are extensively conjugated and metabolised during absorption in the small intestine and then again in the liver. There is particularly strong evidence for extensive phase I de-glycosylation and phase II metabolism by UDP-glucuronosyltransferases, sulphotransferases and catechol-*O*-methyltransferases to yield glucuronides, sulphates and *O*-methylated derivatives [44, 204]. Indeed, in the small intestine's jejunum and ileum there is efficient glucuronidation of nearly all polyphenols to a greater or lesser extent by UDP-glucuronosyltransferase enzymes. Olive oil simple phenolics hydroxytyrosol and tyrosol are also extensively conjugated to glucuronides in both the jejunum and ileum tracts of the small intestine [218]. Hydroxytyrosol, as a catechol, is also subject to extensive *O*-methylation by the action of catechol-O-methyltransferase. Unabsorbed polyphenols will reach the large intestine and be further metabolised to simple phenolic acids by enzymes of the gut microflora.

b) Lower GI tract

Studies have suggested relatively small amounts of absorption of dietary polyphenols in the small intestine, to the extent of just 10% to 20%, which implies that most ingested polyphenols, including those absorbed and conjugated in the enterocytes and/or the liver before they are returned directly or through the bile, progress as far as the large intestine and the colonic microflora [227-230]. With some 10^{12} micro-organisms/cm^3, the colon has huge catalytic and hydrolytic potential, and the enzymatic degradation of flavonoids leads to the creation of a huge

array of new metabolites. Bacterial enzymes, for example, can catalyse a number of reactions including hydrolysis, dehydroxylation, demethylation, ring cleavage and decarboxylation as well as rapid de-conjugation [231]. In the large intestine bacteria catalyse the breakdown of the flavonoid backbone itself to simpler molecules such as phenolic acids. Specific metabolites have been found in urine after a range of phenolics have been ingested. For example, hippuric acid, the glycine conjugate of benzoic acid, is mainly derived from plant phenolics and aromatic amino acids through intestinal bacteria action and hippuric acid levels would, therefore, be expected to rise in the urine of individuals consuming diets rich in flavanols or polyphenols. However, hippuric acid could also be derived from other sources such as quinic acid or the aromatic amino acids tryptophan, tyrosine and phenylalanine, or from the use of benzoic acid as a food preservative.

It is believed that the 5,7,3,3′,4′-hydroxylation pattern of flavan-3-ols enhances ring opening after hydrolysis [232] and metabolism of flavanols by enzymes of the microflora of the large intestine result in many metabolites: 3,4-dihydrophenylacetic acid, 3-hydroxyphenylacetic acid, homovanillic acid and their conjugates derived from the B-ring and phenolic acids from the C-ring [231]. Flavanols, because of their structures, they lack a C-4 carbonyl group, can also degrade to the specific metabolites phenylvalerolactones. Phenylpropionic acids, which may undergo further metabolism to benzoic acids, may also, as shown by animal studies, be the products of flavanol metabolism, which demonstrates fission of the A-ring [231]. The metabolism of flavan-3-ol oligomers may also take place in the colon. A single ingestion of green tea has been enough to detect colonic-derived metabolites of flavanols in human plasma and urine, suggesting significant metabolism by gut microflora in the colon [233]. Such flavonols as quercetin-3-rhamnoglucoside and quercetin-3-rhamnoside may also undergo metabolism by the colonic flora with *Bacteroides distasonis*, *B. uniformis* and *B. ovatus* able to cleave the sugar using α-rhamnosidase and β-glucosidase to liberate quercetin aglycone and other phenolic metabolites [234, 235]. Other bacteria, such as *Enterococcus casseliflavus*, have been found to degrade quercetin-3-glucoside [236], luteolin-7-glucoside, rutin, quercetin, kaempferol, luteolin, eriodictyol, naringenin, taxifolin, and phloretin [237] to phenolic acids and *E. ramulus* is capable of degrading the aromatic ring system of quercetin producing the transient intermediate, phloroglucinol [231]. Other flavonoid glycosides, hesperidin, naringin and poncirin are also metabolised to phenolic acids, via aglycones, by human intestinal microflora that produce α-rhamnosidase, exo-β-glucosidase, endo-β-glucosidase and/or β-glucuronidase enzymes [238]. Studies have shown that 99.9% of anthocyanin glucosides in foods are unabsorbed in the upper GI tract and reach the colon, suggesting extensive bacterial biotransformation. Anthocyanin metabolism by colonic microflora and the formation of high amounts of small phenolic acids such as protocatechuic and syringic

acid as degradation products has been proved, suggesting that phenolic acids could be the more bioavailable form after intake of anthocyanins [239, 240].

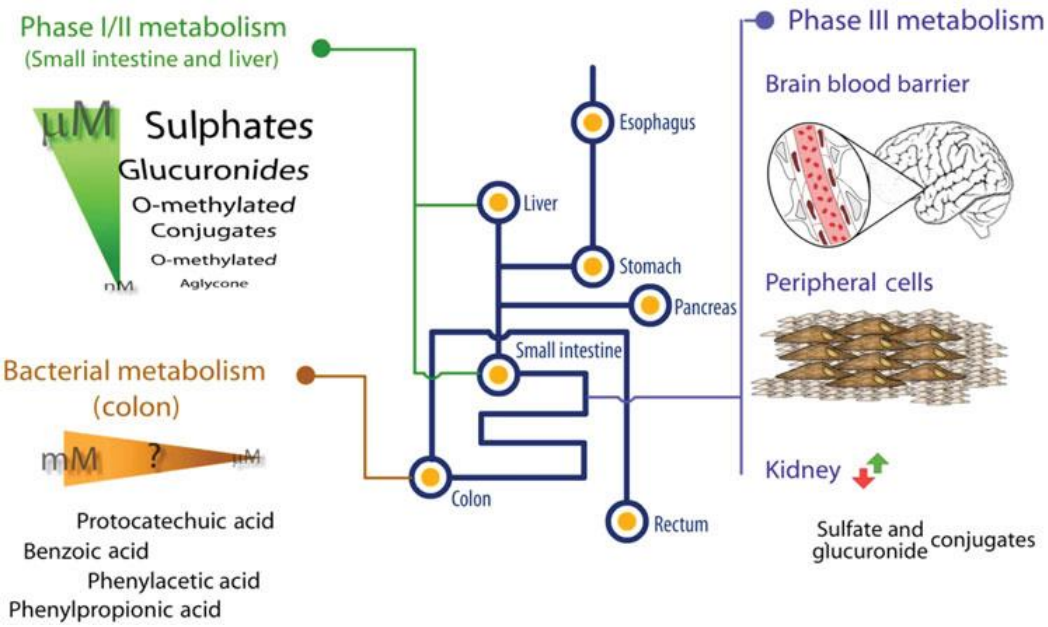


Figure 1.8 The metabolic pathway of dietary polyphenol: flavonoid in human subjects.

In phase I/II metabolism most flavonoids are released from food gradually through the action of digestive juices and colonic microflora in the gastrointestinal tract. Phenolic compounds are delivered into the blood stream and eventually some enter particular tissues/cells in phase III metabolism Adapted from Rendeiro,2012 [5].

1.4 POLYPHENOLS AND CVD

1.4.1 IMPACT OF POLYPHENOL INTAKE ON CVD

Hypertension is a major cardiovascular disease risk factor and is associated with the development and progression of atherosclerosis. For the past two decades, polyphenolic compounds have been studied for their health benefits in preventing and treating a number of diseases, but particularly CVD [47, 50, 241, 242]. Clinical and nutritional epidemiological studies have shown that populations with diets rich in polyphenols are less susceptible to chronic CVDs, including to systemic hypertension, and have lower morbidity and mortality [181, 242-244]. Polyphenols such as resveratrol, epigallocatechin gallate (EGCG), and curcumin, have been shown to have beneficial effects on cardiovascular health [245, 246]. The therapeutic efficacy of polyphenols appears to be associated with average daily dietary consumption, polyphenol type, bioavailability in terms of mode of absorption, tissue penetration, plasma concentration and routes of elimination [247].

Potential health benefits may be partly attributed to the natural antioxidants present in polyphenol-rich foods, such as tocopherols, ascorbic acid and carotenoids, as well as their phenolic compounds (polyphenols) *per se* [248]. Previously, these benefits were thought to be attributable to the antioxidant capacity of polyphenol molecules afforded by multiple ring structures [58, 249-251]. However, recent evidence suggests that concentrations of polyphenols in the circulation are unlikely to elicit health benefits through antioxidant activity alone, because they are absorbed and metabolised rapidly and, therefore, lose their antioxidant properties. It is probable, therefore, that they have other effects on cells, independent of antioxidant mechanisms, which may be through actions on the RAAS and/or the NO systems [252-256].

Numerous randomised controlled trials (RCTs) have demonstrated the beneficial effects of polyphenols on cardiovascular health [42, 49, 257-260]. An inverse association has frequently been demonstrated between the consumption of foods high in polyphenols and the occurrence of hypertension [57, 261, 262]. This antihypertensive effect is thought to be due to the protection and enhancement of endothelial functions, and may be related to inhibition of RAAS enzymes, and increased NO bioavailability [29, 46, 162, 167, 174, 263-267]. The evidence for the role polyphenols might play in maintaining vascular health depends on appropriate markers of peripheral vascular function; assessment of endothelial function is often favoured, as it can identify endothelial damage and predict cardiovascular risk [268]. It should be possible to describe disease development and to obtain prognostic data in advanced phases of CVD from measurements of endothelial function [269].

Very few well-controlled clinical studies have been conducted to date into the impact of flavonoids on CVD mortality, many more human intervention studies, all carried out short-term and on small samples, have examined their effects on CVD risk factors, including vascular function and hypertension. Notwithstanding the noted limitations, when added together the sum of the trials gives credibility to the idea that endothelial function may be improved by flavonoids.

1.4.1.1 EPIDEMIOLOGICAL EVIDENCE

There is evidence from epidemiological studies that flavonoids taken daily may protect humans from cardiovascular illness, but the difficulties surrounding measurement of intake of flavonoids in the human diet must be considered before reaching any conclusions. Three factors make such measurement difficult and the analysis of results from such studies problematic. The first is simply the absence of comprehensive and systematic information regarding the amount of flavonoids in commonly consumed foods. The USDA flavonoid database and Phenol Explorer, major resources in this field, are known to be flawed because of the variation in properties (for

example variety and ripeness) and growing conditions of the plants from which foodstuffs were prepared for assessment [270]. The second is finding a method of analysis that can measure flavonoid intake biomarkers in human samples. Thirdly is the absence of information on what impact food processing has on food flavonoid content and it is likely that the flavonoid content of processed foods is often overstated.

Thirteen of fifteen studies in a review of flavonoid intake and its relationship with cardiovascular disease showed a positive correlation between intake of flavanols and flavanones and the risk of CVD, mortality was reduced by up to 65% [271]. Positive results were also forecast by the Iowa Women's Health Study of 34,489 postmenopausal women carried out over sixteen years, which found a correlation between anthocyanin and flavanone intake and reduced CVD-related mortality [52], foods highlighted in this study as most likely to significantly contribute to reduced risk of CHD and CVD risk were bran, apple, pear, chocolate, red wine and strawberries [259].

The Zupthen Elderly Study followed 806 elderly men for fifteen years and found an inverse correlation between flavonoid intake and risk of death due to ischaemic heart disease. Cocoa, which is rich in flavanols, when ingested was shown to be associated with reduced CVD, lower BP and total mortality [272-274]. Tea is another rich source of flavanols and meta-analysis has shown that three cups each day can reduce the risk of cardiovascular illness by about 11% [275]. Reduced risk of CVD has also been shown to result from red wine intake [276], and, in a systematic review, from ingested soy and cocoa flavonoids [277].

Many foods most strongly associated with reduced CVD risk contain flavanols. The Kuna Indians of the San Blas Islands (Panama) have a diet very high in flavanol-rich cocoa, and studies that have compared them with genetically similar communities in Panama City have shown much lower levels of hypertension and cardiovascular disease among the island people, which is likely to be at least partly due to their very high unprocessed, high flavanol cocoa intake [278]. All of this evidence supports the view that foods like cocoa which are rich in flavonoids may prevent hypertension related to age and therefore CVD.

For all these positive studies, a number of epidemiological studies have found no relationship between intake of flavonoids and risk of CVD [279-282]. This discrepancy may result from shortcomings in diet questionnaires and food composition tables which fail to give an accurate picture of human flavonoid intake; it may also be due to differences between the samples in the different studies, their degrees of nourishment, for example, or their flavonoid intake at the baseline [283]. To avoid these differences, observational studies should in future use reliable biomarkers from plasma, urine or stool samples to assess flavonoid intake.

1.4.1.2 CLINICAL AND IN VITRO EVIDENCE

Markers of polyphenol health benefits include Blood Pressure and RAAS, flow-mediated dilatation (FMD), laser Doppler iontophoresis (LDI) and NO, there is evidence from both humans and animals from intervention trials and *in vitro* studies for the beneficial effects of increased polyphenol in- or uptake.

a) Blood Pressure and RAAS

A number of clinical intervention studies have tested the effects of flavonoid-rich foods on BP. Data from randomised clinical trials is in broad agreement with the results of epidemiological studies [277, 284-292].

Chocolate or cocoa rich foods which are rich in flavanols are widely reported to reduce mild to severe hypertension in both healthy subjects and those at risk of CVD [277, 284, 286, 288, 289]. A meta-analysis of five studies showed dark chocolate consumption reduced systolic BP by 0.7 mm Hg and diastolic BP by 2.8 mm Hg [289]. Meta-analysis of 10 randomised controlled trials of 297 subjects who included healthy normotensive, pre-hypertensive and subjects with stage 1 hypertension found SBP and DBP were reduced by 4.5 mm Hg and 2.5 mm Hg respectively as a result of consuming chocolate or cocoa rich in flavanols for two to eighteen weeks [284]. N.B., however, seven of the studies used white chocolate as a control and the composition of white chocolate is so different from dark chocolate that volunteers could not be blinded. Flavanols in cocoa varying from 36 - 902 mg administered to 49 overweight individuals, reduced BP and improved endothelial function, and may, the authors concluded, therefore, be useful in reducing cardiometabolic risk factors in obese individuals [293].

Studies with polyphenols in green and black teas and coffee have similarly demonstrated a wide range of cardiovascular benefits. A -controlled trial in healthy volunteers found that a daily dietary dose of 400 mg of chlorogenic acid (equivalent to the caffeine content of 2 cups of coffee) resulted in significantly increased plasma concentrations of chlorogenic acid, and, despite the absence of significant changes in NO status and endothelial function markers, there was an observed reduction in post-treatment mean SBP and DBP [255].

Many other foodstuffs containing polyphenols have also been found to be efficacious at reducing BP including numerous berries, however, the evidence, is often from small or short trials and therefore not conclusive. A study that followed 156,957 subjects for fourteen years found that regular anthocyanin consumption, mostly from blueberries and strawberries, reduced hypertension by 8% [42]. Olive oil polyphenols at 30 mg/day in the diet of young women with

mild hypertension led to SBP 7.91 mm Hg and DBP 6.65 mm Hg lower, with a significant increase in plasma NOx (+4.7 ± 6.6 µmol/L, $P < 0.001$) and improved endothelial function [294]. The impact of polyphenol-rich berry juice on BP in a 12-week trial of hypertensive subjects with controls and it was found that with an intake of 500 mL/day, SBP was significantly reduced [295]. Grape polyphenols have been shown to reduce BP and improve endothelial function in a 30-day trial of men at high risk of CVD [296]. Some effects on the RAAS have been demonstrated from polyphenol intake, however, the number and extent of human studies is relatively few: just four studies and all are similarly inconclusive. A supplementary black chokeberry preparation given to patients with metabolic syndrome for two months revealed it as a weak ACE inhibitor [297]. Pomegranate juice containing polyphenols, consumed at 50 ml × 1.5 mmol daily, by hypertensive patients, demonstrated a 36 % reduction in serum ACE activity [254]. The plant polyphenol quercetin was found to be associated with a reduction in BP, however, the reduction was noted independently of changes in ACE activity [298] and blueberry reportedly led to no significant changes in ACE activity, following a 3 week chronic trial [299]

Despite the plethora of studies, insufficient conclusive evidence exists that polyphenols lower BP, largely because there have been insufficient well-controlled, long-term intervention studies. Acute BP reductions after polyphenol intake are widely recognised, but clinical relevance makes it necessary also to observe sustained changes after chronic ingestion over months or years. However, despite the body of evidence, the case for polyphenolic health benefits is not proven, a meta-analysis by Hooper and colleagues showed that chronic intake of black tea, red wine and grapes had no significant effect on SBP or DBP [300-302].

b) Flow-Mediated Vasodilation (FMD)

FMD measures the extent to which arteries are able to vasodilate in response to endothelial NO release in reactive hyperaemia, after the brachial artery has been occluded for five minutes by means of a BP cuff [303, 304]. FMD was first tested *in vivo* by Celermajer *et al.* [305] who used ultrasound to measure brachial artery changes and has been used more recently as a primary outcome by a number of researchers studying the effects of dietary interventions [186, 277, 306, 307]. Most have investigated the acute (within 4 to 8 hours) effect on endothelial function of consuming flavonoid-rich food in healthy subjects or in patients at risk of cardiovascular disease. FMD was used, notably using controlled interventions, to demonstrate that consuming cocoa improved endothelium-dependent vasodilation by increasing the bioavailability of plasma NO in patients with hypertension, CAD, or diabetes; the intervention comprised a cocoa drink rich in flavanols or a control drink with exactly the same macro- and micro-nutrient content but, while

the control drink contained less than 10 mg flavanols the other contained 176 mg of flavanols (monomers and oligomers) [308]. Vascular improvement was correlated in time with changes in plasma flavanol metabolites, which suggested that the flavanol/vascular improvement relationship is one of cause and effect. It has also been shown that flavanol-rich cocoa reverses endothelial dysfunction in smokers, and this has been observed, too, in healthy volunteers after short-term consumption of chocolate rich in flavanols [285, 309]. Flavanols appear to be the directly mediating agent in these effects, as ingesting pure epicatechin or epigallocatechin gallate has produced similar vascular effects in healthy volunteers as well as in in patients with CAD [310, 311].

Increased FMD response in healthy volunteers and CVD patients has been reported after consuming other foods rich in flavonoids, including black tea, grape juice and red wine [312-318]. Consuming grape juice for fourteen days has produced a long-term vascular effect in CAD patients, while chronic intake of flavanol-rich cocoa increases FMD response and hyperaemic brachial artery blood flow in hypercholesterolemic postmenopausal women [319, 320]. Fourteen days consuming flavanol-rich chocolate by hypertensive subjects also increased FMD [286]. Finally, it has been shown that long-term tea consumption produces sustained increase in baseline FMD levels and an additional increase in FMD response [312]. This has also been noted in both healthy smokers and diabetics with high flavanol cocoa [321, 322].

The most convincing evidence so far amassed that endothelial function is improved by polyphenols derives from foods rich in flavanols, especially cocoa [44]. Meta-analysis of 42 human trials comprising a total of 1297 participants found that FMD improved after ingestion of chocolate/cocoa in both acute and/or short-term chronic formats, while another found that a 2.6% improvement in FMD is possible after moderate consumption (500 ml or 2 to 3 cups per day) of green or black tea [323, 324]. Improved FMD response has also been noted in relation to other foods rich in polyphenols. Meta-analysis of nine studies found that FMD improved significantly 30, 60 and 120 minutes after consuming grape polyphenols but did not report any effect after chronic interventions [241]. It was demonstrated that two weeks of consuming 472.8 mg of TPs (total polyphenols) from Concord grape juice improved healthy smokers' FMD, while another chronic study found that, for men with metabolic syndrome, consuming 267 mg of TPs of a grape preparation for thirty days significantly improved vascular endothelial function [296, 325].

The evidence of FMD improvement in the case of berry phenolics is not so persuasive. No cumulative effect was found from chronic cranberry juice consumption at the rate of 835 mg TPs and 94 mg anthocyanins, either on FMD or on vascular function in general; the sample comprised

stable CAD patients [326]. Notwithstanding that, the same authors carried out an open-label pilot study which found the effect on FMD to be acute and favourable, while a different study found that 766 mg of TPs, including 310–727 mg anthocyanins, in a blueberry drink consumed by healthy young men gave significantly and acutely improved FMD 1, 2 and 6 hours after consumption [327]. This acute effect was also visible when processed blueberry products were consumed by matched volunteers; this suggests that the form in which polyphenol-rich blueberry is consumed is not relevant to the benefits conferred [328]. In the case of overweight men, an acute study showed a correlation between improved FMD and consumption of 694 mg TPs, including 493 mg anthocyanins, in açaí [329].

The flavanone content of citrus fruits may also positively affect endothelial function. Vascular impairment in healthy middle-aged men, as measured by FMD, was reversed by acute flavanone intervention, whether as whole orange or as juice [330]. The dose was 128-452 mg TPs, including 107-352.80mg hesperidin, and the effect was observed notwithstanding the differing levels of flavanone metabolites measured in the subject's plasma. It has also been shown that a 2.5% FMD improvement is experienced by subjects with metabolic syndrome after three weeks of consuming 500 mg of hesperidin per day [331].

An evaluation of FMD of the brachial artery demonstrated that red grape polyphenol extract, which contains a wide variety of polyphenolic compounds, could trigger a surge in FMD, showing an acute improvement in endothelial function [332]. Clinical assessment of post-ischaemic endothelial function of the brachial artery, following black tea doses of 5 cups per day over a 4-week-period, demonstrated significant and consistent FMD [333]. In acute, uncontrolled pilot studies, it was demonstrated that mean brachial artery flow was enhanced 4 hours post consumption of a single 480 mL dose of cranberry juice [334]. Using double-strength cranberry juice (54% juice, containing 835 mg TPs, and 94 mg anthocyanins) per day over a 4-week period, the same authors also found, in patients with CAD, reduced mean carotid-femoral pulse wave velocity, a measure of central aortic stiffness. It has also been found that polyphenol consumption in both blueberries (at 1,791 mg) and cranberries (at 1,910 mg) led to significant increases in FMD at 1-2 and 6 hours post-intake [49, 335]. Onion peel extracts containing 162 mg quercetin consumed daily for a 12-week period, enhanced endothelial function, as defined by FMD and endothelial progenitor cell (EPC) circulation measured by flow cytometry, in healthy obese individuals [336].

c) Laser Doppler Iontophoresis

Another commonly used marker for reduced risk of CVD is LDI, which measures vasodilation of the forearm's peripheral microvasculature in response to 1 % acetylcholine (Ach) (endothelium-dependent vasodilation) and 1 % sodium nitroprusside (SNP) (endothelium-independent vasodilation) agents delivered by iontophoresis using an electrical field. A laser Doppler imager measures changes in blood flow and the area under the curve (AUC) and incremental area under the curve (iAUC) express microvascular responses for flux versus time, using arbitrary units of measure [337].

Studies using laser Doppler flowmetry (LDF) have shown that endothelial dysfunction in patients with preclinical and borderline hypertension can be improved by pycnogenol extract, which abounds in water-soluble polyphenols including procyanidins, bioflavonoids and organic acids [338]. In another trial, oral administration of pycnogenol, was also shown to enhance NO production leading to improved forearm blood flow by elevating levels of endothelium-dependent vasodilation in response to Ach [339].

In a randomised control trial, LDI was used to assess the effects of acute ingestion of a purée-based drink rich in flavonoids and demonstrated improved vascular reactivity and endothelium-dependent vasodilation [340]. It has also been found that acute consumption of a flavonoid-rich blackcurrant juice drink on improved vascular reactivity markers measured by LDI [341]. Another randomised control trial, in this case dose-dependent and concerned with men on a diet high in flavonoids, used LDI to show an improvement in endothelium-dependent microvascular reactivity together with reduced vascular cell adhesion molecules including E-selectin and lower levels of C-reactive protein [342]. Polyphenols in champagne have also been shown to be beneficial with respect to CVD, inducing a significant, positive endothelium-dependent vascular affect within 4 hours [343], while acute consumption of blackcurrant juice, known to be polyphenol-rich, was demonstrated by LDF to produce a positive impact on vascular reactivity markers [341]. Earlier studies using LDF to examine acute effects of cocoa on microcirculation in the dermis also showed an increase in blood flow and oxygen saturation [344].

Animal studies using female, spontaneously hypertensive rats (SHR) have demonstrated a range of cardiovascular effects following consumption of red wine polyphenols. Doxorubicin was prevented from inducing blunted endothelium-dependent hyper-polarisation (EDH) type relaxations and vascular oxidative stress. Expression levels of target proteins were improved. ROS and endothelium-derived contracting factors in Ang II-induced endothelial dysfunction were

counteracted. COX-1 and COX-2 were upregulated. SBP was reduced and the endothelium-dependent relaxation response to Ach improved. [345, 346]

d) Nitric Oxide (NO)

Studies in humans have shown correlations between vascular and BP improvements and increases in circulating NO with flavonoids and metabolites appearing in the plasma, and it is likely that flavonoids influence NO levels [176, 345]. RCTs examining the effects of polyphenols on FMD in humans, considered likely to rely on bioavailability of NO, indicate significant improvements in both acute and chronic cases [346].

A study of (-)-epicatechin's vasodilatory effects in a coca drink rich in flavonols using healthy adult males indicated acutely elevated levels of circulating NO [347]. Hydroxycinnamates and phenolic acids are flavonoids in champagne and have been shown in fifteen healthy adults to raise the bioavailability of NO and acutely improve vascular function [348]. Vanillic and homovanillic acids are among the small phenolic acid metabolites of blueberry flavonoids and are similar in structure to the pharmacologic NADPH oxidase inhibitor apocynin. The conclusion of an acute study of healthy men was that consuming them by way of 766, 1278, and 1791 mg doses of blueberry flavonoids was associated with a reduction in NADPH oxidase activity in neutrophils [349]. Evidence is increasing that polyphenols have a beneficial effect on the bioavailability of NO as well as identifying possible mechanisms, but an exact understanding would require cell and animal studies using physiologically relevant doses as well as well-designed chronic human intervention studies.

1.4.2 INVESTIGATIONS INTO THE UNDERLYING MECHANISMS OF POLYPHENOLS ON VASCULAR FUNCTION

a) RAAS

In *in vivo* studies of humans, food components like pomegranate juice, rooibos tea, green tea and polyphenols (especially flavonoids) have been shown to have inhibit ACE activity, preventing Ang I from being converted into Ang II. Induced reduction in Ang II leads vasodilation and blood volume is increased and BL is lowered [350, 351]. A decrease in the expression of endothelin-1 messenger RNA, increased by Ang II inhibition of endothelin secretion and regulation of the changes in intracellular calcium concentration and mitochondrial membrane potential, have all been demonstrated [352]

In vivo studies have demonstrated that polyphenols regulate the RAAS by reducing plasma Ang II and renin levels, thereby reducing hypertension. For example, an animal model of endothelial

function in aldosterone-salt-mediated hypertension demonstrated a link to an increase in circulating micro particles, the release of which were retarded by polyphenols [353]. Studies of the effects of polyphenols in red wine found that they prevented Ang II-induced VEGF expression and matrix metalloproteinase enzyme (MMP)-2 activity in the aortic wall, and Ang II-induced expression of eNOS, the formation of ROS, and the nitration of protein [354], resulting in reduced hypertension and improved endothelial function. It has been reported that they prevented Ang II-induced hypertension and endothelial dysfunction as shown by vascular relaxation, by normalising vascular superoxide anion production and NADPH oxidase subunit expression in *ex vivo* organs [355]. Another study of red wine polyphenols, on ischaemic rat hindlimbs, showed the angiogenic processes implicated in the development of atherosclerotic lesions, the formation of ROS and nitrated proteins, and the expression HIF-2alpha, eNOS, and VEGF induced by Ang II were prevented [356]. A 4-week study of the mechanisms underlying the amelioration of a range of endothelial dysfunctions in mesenteric artery rings of middle aged rats found the expression of eNOS, ArgI, NADPH oxidase and angiotensin receptors were all normalised by red wine [357].

Recent studies have also shown improvements in the management of doxorubicin-induced vascular injury (damaged endothelial cells, increased vascular permeability) by improving the vascular RAAS. This was assessed by organ chamber vascular reactivity, dihydroethidine detection of vascular formation of ROS, levels of small and intermediate calcium-activated potassium channels involved in EDH-type relaxations and immunofluorescence detection of eNOS, Ang II, and AT1 receptors [358]. *In vitro* studies, with quercetin and wine polyphenols, have shown that flavonoids could regulate the NO-guanylyl cyclase pathway, EDH factor(s), endothelin-1 and protect endothelial cells from apoptosis. Similarly, and in the same review, *in vivo* studies with hypertensive animals have also demonstrated the prevention of endothelial dysfunction, reduction in BP, regulation of oxidative stress and protection against end-organ damage [359].

Studies with polyphenols from green and black teas have shown a decrease in the expression of endothelin-1 messenger RNA, increased by Ang II inhibition of endothelin secretion and regulation of the changes in intracellular calcium concentration and mitochondrial membrane potential [352], this corroborates earlier work, which showed decreased ROS through the regulation of the protein expression of NADH oxidase, p22phox and p67phox, and upregulated catalase expression in bovine carotid artery endothelial cells [360]. Superoxide anion levels and permeable fluorescence intensities were notably decreased in Ang II-stimulated bovine carotid artery endothelial cells, suggesting that tea polyphenols could assuage Ang II-induced hyperpermeability, primarily by the reduction of ROS and regulation of ROS-related protein expression [360]. Similar studies, involving caffeic acid along with its nineteen novel derivatives,

chlorogenic acid and quercetin on the inhibition of renin and ACE activities, demonstrated strong renin (IC_{50} = 229 μM) and ACE (IC_{50} = 9.1 μM) inhibition [361].

Studies on enzyme kinetics also suggest that tea polyphenols inhibit ACE activity. Substrate-dependence of the reaction kinetics for green and black tea polyphenolic size fractions have revealed enzyme velocity curves that matched allosteric and non-Michaelis-Menten relationships, with a mixed mode of *in vitro* inhibition of ACE, mostly of a kinetically uncompetitive type [362]. Combined blackberry (*Rubus fruticosus* agg.), raspberry (*R. idaeus*) and black raspberry (*R. occidentalis*) polyphenol extracts counteracted Ang II-induced senescence in vascular smooth muscle cells. An attempt to decipher the molecular mechanisms underlying the process found that 200 μg mL of the triple berry extract led to a decrease in cells positive for senescence-associated β-galactosidase, and downregulated p21 and p53 expression associated with decreased levels of ROS and Ang II signalling [363]. This study also showed that blackberry polyphenol extract increased superoxide dismutase 1 expression, attenuated the upregulation of NADPH oxidase 1 (NOX1) expression and the Ang II-induced phosphorylation of Akt, p38 MAPK and extracellular-signal-regulated kinase 1/2, as well as reducing senescence in response to NOX1 overexpression.

Inflammatory angiogenesis is a pivotal pathogenic development in atherosclerosis, and is controlled by the proinflammatory enzyme cyclooxygenase (COX)-2 and degrading MMPs, a study on the effects of oleuropein, hydroxytyrosol, resveratrol and quercetin on endothelial cell angiogenic response *in vitro*, demonstrated these polyphenols could reduce inflammatory angiogenesis in cultured endothelial cells, through MMP-9 and COX-2 inhibition [364]. Another study to evaluate increases of Ang II type I receptor (AT1R), cyclooxygenase-2 (COX-2), lectin-like oxidised LDL receptor-1 (LOX-1), prostacyclin/prostaglandin I 2 synthase (PGIS), and thromboxane A2 synthase (TXA2S) by tumour necrosis factor-alpha (TNFα) in hyperglycaemic conditions (30 mM) of human endothelial cells over a short period, and to investigate the regulatory effects of dietary flavonoids on these increases, demonstrated that apigenin, kaempferol, chrysin, and flavone all significantly impeded TNFα-induced LOX-1 expression and that a flavone skeleton was needed to reduce LOX-1 expression by apigenin, the double bond found in its C-ring, and the absence of a third hydroxyl group from its B- and C-rings [365].

Recent animal studies have demonstrated that a flavonoid-rich red wine vinegar beverage could inhibit the RAAS and hence reduce hypertension. A baseline increase in mean BP induced by Ang I (1 microg/kg, i.v.) was attenuated an hour following administration of the beverage, when a significant reduction in serum ACE activity from 39.4+/-1.2 IU/l at baseline to 37.0+/-1.4 IU/l was

also observed [366]. Another animal study conducted to measure the effect of flavonoids from the seeds of *Astragalus complanatus* found a significant reduction (17%) in mean arterial pressure in the high dose group (200 mg/kg) for both renal and SHR. The antihypertensive regulation was attributed to a decrease in plasma concentration of Ang II caused by the flavonoids quantified by radioimmunoassay at 60 minutes post drug administration [367]. More recent animal studies to evaluate the antihypertensive effect of total flavone extracts from Pueraria Radix (the dried root of *Pueraria lobata) in vitro* have also shown that consumption for as short a period as 14 days notably reduced BP in SHR [368]. The flavone extract considerably inhibited ACE and plasma renin activities, depending on the dose thus substantiating other studies which have found flavone mediated regulation of the RAAS.

In vitro, cultured endothelial cells from human umbilical veins incubated with bilberry 25E extract, exhibited significant, dose-dependent inhibition of ACE activity after 10 minutes [369]. Phenolic extracts from the Garden egg fruit (*Solanum aethiopium = S. aethiopicum L.*) were investigated with enzymes α-amylase, α-glucosidase and ACE, and demonstrated strong inhibition of ACE activity in rat lung homogenates *in vitro* [370]. Studies with lentil polyphenols have demonstrated that extracts could ameliorate Ang II-induced hypertension and associated pathological changes including remodelling and perivascular fibrosis in small resistant arteries of the heart and kidneys by up to 30 %, and reduce ROS levels in the aorta by up to 48.9 % [371].

A study to investigate the *in vitro* modulation of the RAAS by polyphenolic extracts from two green leafy vegetables, *Vernonia amygdalina* and *Gongronema latifolium*, showed that they inhibited ACE and renin [253]. This study also demonstrated that the extraction method was significant, since ethanol column fractionations appeared to show higher ACE and renin inhibition than crude acetone extracts. Peach and plum juices containing polyphenols also reduce Ang II in plasma and its receptor Agtr1 in heart tissues and are, therefore, effective as protection against metabolic disorders that promote CVD [372].

Recent studies have shown that phlorotannins, the predominant polyphenols in brown algae, are ACE inhibitors, phenolic extracts of Spiral wrack (*Fucus spiralis)* showed remarkably high ACE inhibition (88.8 ± 2.4%), with a total phenolic content of 156.6 ± 1.4 mg PE/g of dry weight [373]. *In vitro* experimental evidence has previously confirmed that oat polyphenols have strong ACE inhibitory activity, in various hydrolysis conditions [374].

b) NO

Several mechanisms for the interference of polyphenols with NO have been suggested. There is limited evidence that the high antioxidant nature of phenolic groups and their reaction with ROS may be capable of reducing NO breakdown [375-377]. It is also possible that polyphenols or circulating phenolic metabolites including Ferulic Acid (FA) could prevent the breakdown of NO by inhibiting enzymes that generate ROS, including NADPH oxidase, xanthine oxidase and lipoxygenase [375, 378-380].

The ortho-methoxy-substituted catechol of FA is similar in structure to known NADPH oxidase inhibitors such as apocynin, which may explain why it is effective *in vivo* [381, 382] (Figure 1.9).

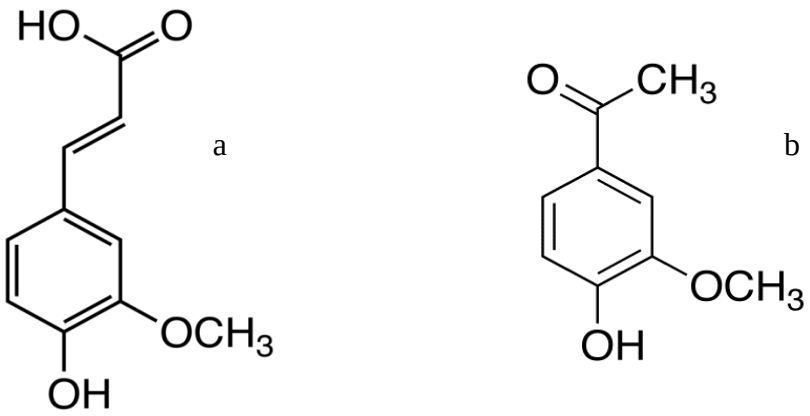


Figure 1.9 Similarities between the ortho-methoxy-substituted catechol structures
Diagram shows (a) ferulic acid and (b) apocynin

eNOS is considered primarily responsible for NO generation in vascular epithelium tissue and a number of studies have considered how it is affected by polyphenols. *In vitro* tests with quercetin and its metabolites administered at 5 and 10 µM, on human endothelial aortic cells [383] have evidenced phosphorylation of eNOS and increased NO production via Adenosine Monophosphate-activated protein kinase (AMPK) and similar outcomes have been found in E-knockout mice [384]. Isolated human arteries have also been investigated, they demonstrated that polyphenols increased endothelial NO formation, mediated by redox-sensitive activation of the phosphatidylinositol 3-kinase/Akt (PI3K–AKT) pathway, again resulting in increased eNOS, activity [385]. Polyphenols were also shown to activate eNOS by raising the concentration of intracellular free calcium, and by activating oestrogen receptors in endothelial cells. A study that examined the effects of (-)-epicatechin, at 0.3-10 µM, on human umbilical vein endothelial cells

(HUVEC) reportedly increased NO, possibly connected with the compound's vasodilatory potential [386]. However, the observed effects of dietary polyphenols *in vitro* do not always translate to an *in vivo* setting. As such, many questions remain concerning their physiological mode of action including the effects of metabolites.

It also thought likely that flavonoids can influence NO levels by modulating endothelial intracellular signalling pathways such as the PI3-kinase/Akt pathway and intracellular Ca^{2+} levels that lead first to eNOS phosphorylation and then to production of NO [46, 176, 308, 387-390]. A supplement of 625 mg/day of pomegranate extract was shown to reduce vascular inflammation and other vaso-beneficial effects by activation of the Akt/eNOS pathway and lower monocyte chemoattractant protein-1 expression [391]. Another recent animal study [392] investigating the effects of chlorogenic acid, a polyphenol abundant in coffee, revealed that it could protect blood vessels against HOCl-induced endothelial dysfunction with a significant increase in NO production [392]. This study also demonstrated that eNOS dimerization was increased, as was the expression of heme oxygenase-1, an isoform of heme oxygenase usually produced during oxidative stress. Another study has demonstrated that polyphenols caused NO-mediated endothelium-dependent relaxations mediated by redox-sensitive activation of the phosphatidylinositol 3-kinase/Akt (PI3K–AKT) pathway, resulting in increased eNOS, increased concentrations of intracellular free calcium and activating oestrogen receptors in endothelial cells may also have been responsible for elevated ENOS levels [385]. Açaí stone extract extracts given to hypertensive rats at 200 mg/kg/day, were also responsible for antihypertensive effects, preventing endothelial dysfunction and vascular structural changes; the mechanisms likely to be associated with antioxidant effects, NOS activation, and inhibition of MMP-2 activation [393]. Studies on rat mesenteric arteries have implicated grape polyphenols in reversing age related endothelial dysfunction that affects both the NO- and EDH-mediated relaxations associated with vascular oxidative stress, as well as activation of the RAAS [394, 395]. Red wine polyphenols, in porcine arterial tissue, induced NO and EDH-mediated coronary vasodilatation, *Aronia melanocarpa* (black chokeberry), an abundant source of polyphenols showed substantial NO-mediated endothelium-dependent relaxations in the same tissue [396, 397].

There is evidence that flavonoids increase eNOS gene expression, inducing production of prostacyclin in endothelial cells and inhibiting endothelin-1 and endothelial NADPH oxidase [398-402]. Inhibition of the latter reduces production of superoxide and its possible scavenging of NO to give $ONOO^-$.

Several other mechanisms of action on NO production have also been proposed following *in vitro* studies. These include endothelin-1 production [403], via both Ca^{2+}-dependent BK Calcium channel-mediated hyperpolarisation, and Ca^{2+}-independent PI3K/Akt pathways [404], redox-sensitive activation of the phosphatidylinositol 3-kinase/Akt (PI3K–AKT) pathway (also resulting in increased eNOS activity). In studies on rat mesenteric arteries, polyphenols in grapes have been implicated in reversing age related endothelial dysfunction that affects both the NO- and EDH-mediated relaxations associated with vascular oxidative stress, as well as activation of the RAAS [394, 395].

Studies *in vitro* have shown that avenanthramides (Avn) inhibited the development of vascular smooth cells, acting against the adverse effects of atherosclerosis [405, 406]. Avn in oats have also been shown to exhibit antioxidant activity in various cells [407]. Avn in enriched oat extract has been shown to significantly suppress IL-1 β-stimulated secretion of proinflammatory cytokines, such as IL-6, IL-8, and MCP-1, by human aortic endothelial cells [408].

Avn-2c was demonstrated to inhibit serum-induced smooth muscle cell proliferation notably at a concentration of 120 μM and to improve NO production, dependent on the dose, in both vascular smooth muscle cells and human, aortic endothelial cells. A three-fold increase in NO production in vascular smooth muscle cells and a nine-fold increase in NO production in human aortic endothelial cells has also been reported [406]. These increases were corroborated by a simultaneous increase in mRNA expression for eNOS, suggesting that Avn could prevent atherosclerosis through inhibition of vascular smooth muscle proliferation and increase NO production in both cell types.

FA increased levels of cAMP, cGMP and phosphorylated vasodilator-stimulated phosphoprotein, while at the same time decreasing phospho-mitogen-activated protein kinase and phosphodiesterase in washed rat platelets [409]. Earlier vasoreactivity studies on ferulic acid, assessed using aortic rings isolated from normotensive Wistar-Kyoto rats (WKR) and SHR, have demonstrated that ferulic acid restored endothelial function through enhanced bioavailability of basal and stimulated NO in the aortas [380]. A similar study to assess the vasoreactivity of ferulic acid in chronic two-kidney, one-clip, renal hypertensive rats, showed that ferulic acid restored endothelial function by altering the bioavailability of NO [410].

However, the observed effects of dietary polyphenols *in vitro* or in animals do not always translate to a human, *in vivo* setting. As such, many questions remain concerning their physiological mode of action including the effects of metabolites. It should be borne in mind, however, that rodents have a very different metabolism to humans.

1.5 POLYPHENOLS OF WHOLE GRAINS

1.5.1 WHOLE GRAIN PHENOLICS

The structure of a representative whole grain is shown in Figure 1.10. The wide range of phytochemicals in whole grain include, *inter alia* folates, tocols, sterols and PAs [411]. The most significant are PAs which comprise derivatives of hydroxycinnamic or hydroxybenzoic acids (Figure 1.11). Most significant among the hydroxycinnamic acids in grain are ferulic, caffeic, *p*-coumaric and sinapic acids, while for hydroxybenzoic acids it is vanillic, syringic, gallic and protocatechuic acids. These PAs are sited primarily in the outer bran layers (Figure 1.10), most abundant are ferulic, *p*-coumaric, vanillic and syringic acids [412]. Small amounts of PAs are available in both free and soluble conjugated forms, but most are bound as complex insoluble esters to polysaccharides [413, 414] and serve as a cross linking mechanism in the plant cell wall. Between 70 % and 90 % of the phenolics found in wheat are ferulic acids (FA) in the grain's aleurone layer, a single layer of cells overlaying the endosperm and adhering strongly to the pericarp (Figure 1.10) [415, 416]. FA is a hydroxycinnamate synthesised from cinnamic and *p*-coumaric acid through the shikimic acid pathway by hydroxylation and methylation reactions [417].

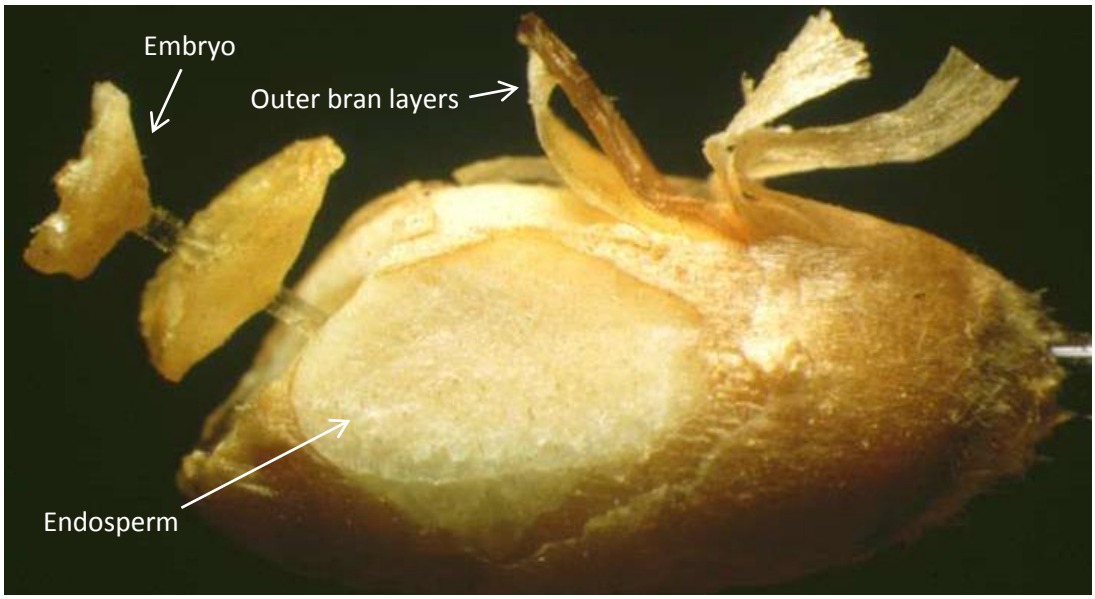


Figure 1.10 Break down (structure) of a typical whole grain.
Most of the micronutrients (including PAs and avenanthramides) are contained in the outer bran layers. In the production of white flour, only the starchy endosperm is used. After Krygier, 1982 [3].

Another constituent of whole grain is diferulic acids, or FA dehydrodimers, which are present in different forms; the main structures involve oxidative coupling at 8-5, 8-O-4, 8-8 and 5-5 in grass cell walls [7]. Monomer and dimer FA forms both bind covalently via ester linkage to the major structural polysaccharide arabinoxylans, as well as to mixed, linked β- glucan and other polysaccharides (Figure 1.11). This attachment is by way of the carboxylic acid group acylating the primary hydroxyl at the C5 position of the arabinosyl side-chains of arabinoxylans (AXs), with the commonest being 5-O-feruloyl-L-arabinofuranose and 5-O-feruloyl-AXe [418]. 95.8% of bran is conjugated form with only 4.2% present in a free form [419]; the antioxidant potential of bran has been shown to be determined by the amount of free FA released during metabolism in the digestive tract.

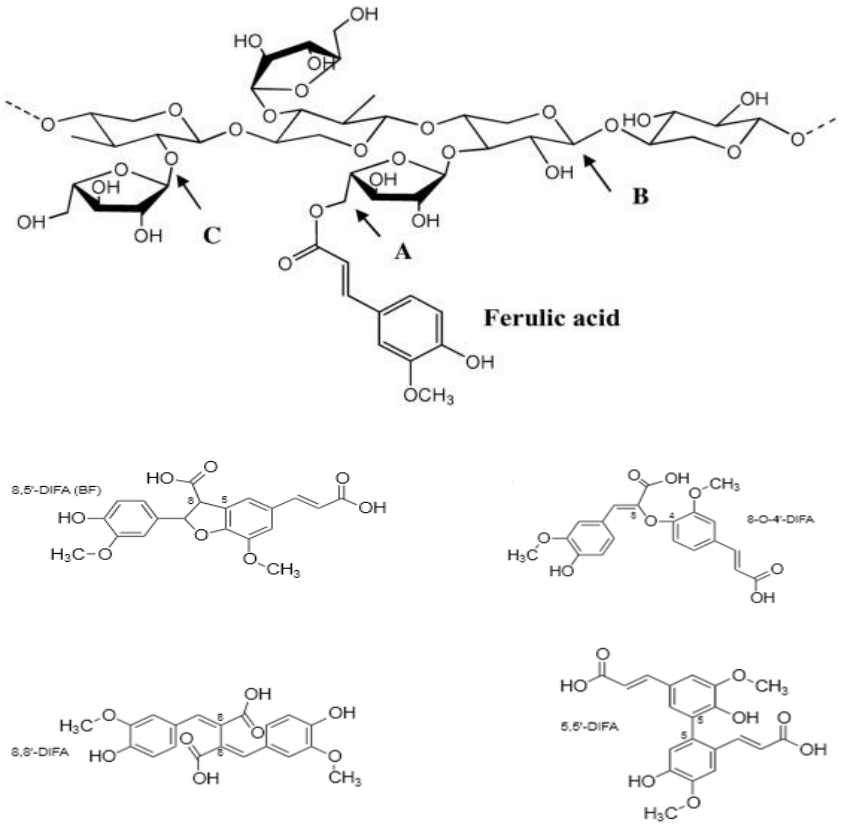


Figure 1.11 Ferulic Acid Esterified to Arabidoxylan

(A) Ferulic Acid linked to O-5 of arabinose chain. (B) β-1,4-linked xylan backbone. (C) α-1,2-linked L-arabinose [1]. The most common diferulic acid structures with oxidative coupling at 8,5, 8-O-4, 8-8 and 5-5. Ralph *et al* 1994 [7].

The oats (*Avena* spp., family Poaceae) are a genus of cereal grains which originated in the Mediterranean region and grow well in temperate regions where some species, particularly *Avena sativa* L., the subject of this study, are widely cultivated. Oat meal is a popular food produced from the seed of *Avena sativa* L. and is chiefly consumed in the form of porridge which comes in

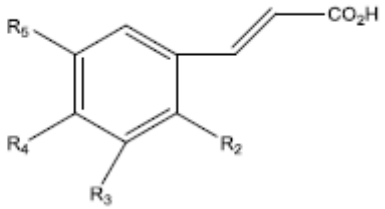
several varieties, including steel cut oats, rolled (also known as old fashioned and whole) oats, and quick cook (also known as instant) oats. The varieties derive from increasing degrees of processing, which results in faster cooking times and substantially different nutrient compositions [420]. The widely established health benefits of oats have led to an increase in their consumption in recent years [421]. Oats are a rich source of soluble fibres, β-glucans, which contribute to the reduction of blood cholesterol [422], and also contain a unique group of low molecular weight soluble phenolic compounds, the avenanthramides which are not present in other cereals [406]. Avenanthramides have recently been shown to exhibit anti-inflammatory, anti- proliferative and anti-itching properties which may provide protection against CHD, colon cancer and skin irritation [407, 436].

Class of Nutrients	**Nutrient Components (%)**	**Location in the oats**	**References**
Starch	Amylose 60 %	Endosperm	[423-426]
Protein	Total 11 - 15 % Globulins (% of total) 80 % Prolamins 15 % Glutelin 5 - 66 % Albumin 1 - 12 %	Germ and bran	[427, 428]
Lipids	Total 5 - 7 % (mono and polyunsaturated fatty acids: mainly oleic (18:1) and linoleic (18:2) saturated fatty acids including myristic (14:0) and palmitic (16:0) acid)	Kernel and bran	[429, 430]
Trace Minerals	Calcium: 0.54 % Iron 0.04 - 7 %	Bran	[431]
Vitamins	Niacin 0.032 % Thiamine 0.00 2 % Riboflavin 0.001 %	Bran	[431]
Fibre	Total: 10-12% β-glucan 2.3 - 8.5 %	Bran	[432, 433]
Phytochemicals	Tocols α-Tocotrienols and α-tocopherols (% of total) 86 - 91 % Phenolic Compounds 5.7 % (Ferulic, Vanillic, Syringic, Protocatechuic, *p*-Coumaric, Caffeic, Sinapic) Flavanoids trace % Avenanthramides AV-A 2.1 - 4.3 % AV-B 2.8 - 6.2 % AV-C 2.5 - 4.7 %	Bran and outer layers of Kernel	[434, 435]

Table 1.1. Nutritional composition and disposition of Oats (*Sativa* spp.)

Oats are also a rich dietary source of phytochemicals including vitamin E, phenolic acids and avenanthramides [437]. Phenolic acids contain one aromatic ring bearing acid group and one or more hydroxyl groups (Figure 1.12) [438], they are found in three different forms within the oat food matrix: as soluble free acids; as soluble conjugates esterified to low molecular weight components such as sugars; and as insoluble bound acids esterified to high molecular weight components including lignin, cell wall polysaccharides and storage proteins [439, 440]. Hydroxybenzoic acids and hydroxycinnamic acids are the two classes of phenolic acids found in oats. Hydroxybenzoic acid derivatives include, protocatechuic, syringic, vanillic, *p*-hydroxybenzoic and gallic acids, while hydroxycinnamic acid derivatives are ferulic, *p*-coumaric, *o*-coumaric, caffeic and sinapic acids (Figure 1.12) [441, 442]. Avenanthramides are a unique group of compounds in oats, consisting of an amide conjugate of anthranilic acid and hydroxycinnamic acids. The 3 major subgroups are avenanthramide-A (Avn-A), avenanthramide-B (Avn-B) and avenanthramide-C (Avn-C), they all occur in the bran or outer layers of the kernel (Figure 1.12).

The chemical structure of Avn and its subgroups is shown in Figure 1.12. Avn-C is one of the major avenanthramide polyphenols of oats, it inhibits the serum-induced proliferation of vascular smooth muscle cells, a vital process in the onset of atherosclerosis. Flow cytometry analysis on this inhibition process has shown that Avn-C could block the cell cycle in G1 phase, as a rise in the number of G1 phase cells and a fall in the number of S phase cells was observed. Inhibition of the cell cycle along with decreased cyclin D1 expression and increased cyclin-dependent kinase inhibitor p21cip1 expression, with no notable changes in p27kip1 expression, have also been linked with Avn-c mediated reduction in the phosphorylation of retinoblastoma protein, a key process in G1-S transition [405].



Cinnamic acid derivatives

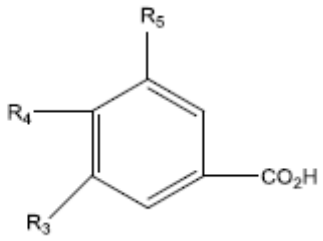
R_2=OH, o-coumaric acid

R_4=OH, p-coumaric acid

R_3=R_4= OH, caffeic acid

R_3=OCH_3 , R_4 =OH, ferulic acid

R_3= R_5 =OCH_3, R_4= OH, sinapic acid



Benzoic acid derivatives

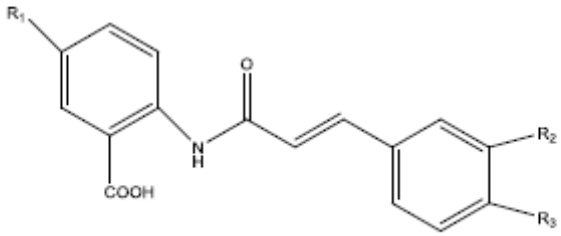
R_3 =R_4 = R_5=OH, gallic acid

R_4 =OH, p-hydroxybenzoic acid

R_3 =R_4 =OH, protocatechuic acid

R_3=OCH_3 , R_4 =OH,vanillic acid

R_3= R_5 =OCH_3 , R_4= OH, syringic acid



Avenanthramides

R_1 = R_3=OH, avenanthramide-A

R_1 = R_3=OH, R_2 =OCH_3,avenanthramide-B

R_1 =R_2= R_3=OH, avenanthramide-C

Figure 1.12 Chemical Structure of Cinnamic Acid derivatives, Benzoic Acid derivatives and Avenanthramides A-C in Oat.

1.5.2 BIOAVAILABILITY OF WHOLE GRAIN PHENOLICS

While studies involving animals indicate immediate and effective absorption of free FA in the stomach [419, 443, 444], the large and complex nature of esterified diferulates means that they cannot be absorbed through the mucosal barrier. Free FA absorption has been reported in the small intestine in direct proportion to the amount perfused [445-447], but molecules bigger than 30 kDa cannot diffuse through the small intestinal mucosa, which suggests that the potential for absorption of FA and diferulates in the small intestine depends on its initial release from the bound state [448]. While there have been reports of esterase activity in the small intestine's mucosa and to a low extent (0 – 5 %), in the lumen [445, 449], only about 2.6% of total feruloyl molecules are released during gastric and small intestine digestion [450]. This is supported for

both wheat and rye that, in both human and rat intestines, esterase(s) induces release of free diferulic acids and monoester products via hydrolysis of 5-5, 8-O-4 and 8-5-benzofuran diferulate ester bonds [445]. It is known that most FA-AX cleavage occurs in the colon during fermentation in which faecal bacterial esterase activity releases 20% of AX linked hydroxycinnamic acids from bran [449]. Esterase activity, i.e. hydrolysing diferuloyl ester bonds, is carried out more efficiently by human colonic microbiota than by those in the small intestine, due to the presence in large intestinal bacteria of other enzymes and in particular xylanases which can begin the breakdown of insoluble fibre improving the access of esterases to FA-AX complexes. Esterase on its own releases only 4% of alkali-extractable FA from wheat bran; when xylanase is also present the figure rises to 95% [451].

When insoluble AX reaches the colon, xylanases cleave the xylan β-1,4 backbone by to release small soluble oligosaccharides which make easily accessible good substrates for ferulic acid esterases [452]. These in turn release free FA, the amount of which is influenced by the xylanase's source. *Talaromycetes emersonii* xylanases release 1.35% of total FA while for *T. viride* xylanase the figure is 48 % [453]. This difference results from the rate of polysaccharide degradation and the xylanase's ability to produce feruloylated oligosaccharides with longer chain lengths of up to 3 or more units, the optimum size for esterase action [452, 454].

Few human studies have focused on FA absorption and metabolism after consumption of whole grain wheat. Maximum hydroxycinnamate absorption from high-bran cereal, a particularly rich source of FA and diferulates because they include the outer husk including the aleurone layer, occurs between one and three hours after ingestion [455]. It was not found necessarily to be the case, though, that the main cleavage and release of FA from AX happens in the small intestine, but more likely that FA observed in plasma derived from the 4 % of free FA that the grain also contains. It also found low hydroxycinnamic acid levels in plasma after 6 hours, which suggests not much absorption occurring in the large intestine. This may be the result of long, slow FA cleavage as food passes through the large intestine, with minimal amounts being observed in the circulation. No diferulic acids were detected in urine or plasma and were most likely still in bound form, which would suggest that very little of these compounds was absorbed all the way through the gastrointestinal tract. All of this suggests the need for further human studies to fully understand absorption and metabolism of whole grain phenolic acids.

While cleavage and release of ferulic and diferulic acids from wheat bran can take place in the human gastric tract, how far this can extend is unclear. The gut's acidic environment may have

the ability to cleave ester bonds, with the ester cleaved by acid hydrolysis to yield a carboxylic acid (R-COOH) and an alcohol (Figure 1.13) [456].

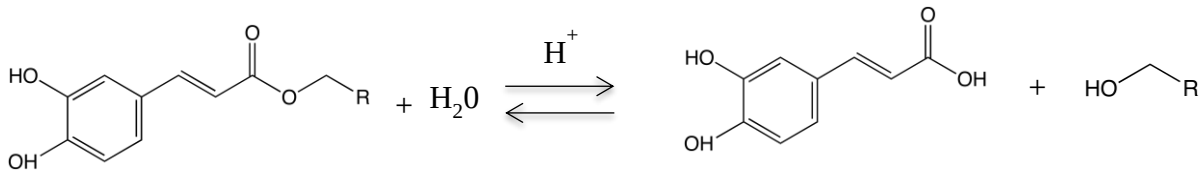


Figure 1.13 The ester to ferulic acid cleavage reaction

Gastric acid is thought to catalyse the ester bond along with H_2O, resulting in free ferulic and carboxylic acids. After Hilal *et al.* 2006.

FA is recovered intact after being incubated in HCl-NaCL (9g/L) aqueous solution for one hour at 37°C [444], this suggests that the acid nature of the gut breaks it down or metabolises it very little. Alkaline, as opposed to acidic, hydrolysis causes release of PAs, including FA, in markedly greater amounts [457], hence it follows that using both esterases and xylanases in bread making may make these hydroxycinnamates more bioavailable, increasing potential health benefits.

1.6 WHOLE GRAIN AND VASCULAR FUNCTION

1.6.1 EPIDEMIOLOGICAL STUDIES

A number of studies have shown an inverse relationship between CVD and whole grain intake as well as positive links between whole grain and whole grain product intake and higher insulin sensitivity, lower waist to hip ratio, lower cholesterol, lower LDL and total lipids, and lower fasting insulin concentrations [458-464]. The Nurse's Health Study followed 75,521 women between the ages of 38 and 63 for 10 years, looking for a relationship between heart disease and whole grain intake [465]. The sample excluded anyone previously diagnosed with cardiovascular disease such as stroke, angina or myocardial infarction and age and adjusted for smoking and dietary factors such as multivitamin supplements. The results showed a strong inverse relationship between CVD risk and whole grain intake, however, when dietary fibre, folate, vitamin B6 and vitamin E had been adjusted for, the remaining reduction in CVD risk, although slight, was considered likely attributable to other constituents of whole grain. Data from the Iowa Women's Health Study of 34,492 postmenopausal women followed up over eight years supported this conclusion, with a finding that a single serving of whole grain was responsible for a one-third reduction in risk of death from ischaemic heart disease [458]. Here again, adjusting for dietary fibre intake did not change the reduction in risk. Meta-analysis of studies in this area found consumption of 2.5,

compared to 0.2, servings of whole grain per day were associated with a 21 % decreased risk of CVD, as a result of which a number of guidelines were drawn up with the aim of increasing intake of whole grain [466]. The advice most commonly accepted recommends daily consumption of three servings of whole grain products (48g) [467]. While these data do show a positive link between protection against CVD and whole grain intake, they are subject to a number of flaws: food intake is self-reported by trial subjects; food is grouped into refined or whole grain products; and those with a high whole grain intake tend not smoke less, to exercise more and to be more likely to have a higher vitamin diet; whole grain-rich diets tend to be high in carbohydrates, folate and protein and low in fat, cholesterol and alcohol [459].

1.6.2 CLINICAL AND *IN VITRO* STUDIES

Physiological effects suggested to arise from whole grain intake include: lower risk of CHD [468], reduced LDL and cholesterol [469], increased insulin sensitivity [470, 471] and reduced BP [472]. The phytosterols, complex carbohydrates, fibre, minerals and vitamins (especially vitamin E) and polyphenols in whole grain are believed to be responsible for these beneficial effects. They are found for the most part in the bran layers on the surface of the grain (Figure 1.10), which is why refining the grain, leaving for consumption only the starchy endosperm, results in less potential for positive health effects [473-477]. Whole grain is a source of plant sterols and stanols capable of lowering cholesterol owing to their ability to inhibit cholesterol absorption from the small intestine. Studies have demonstrated that increasing dietary phytosterols can increase excretion and reduce cholesterol absorption, so that there is an inverse relationship between total plant sterol intake and total and LDL serum cholesterol [478]. Substituting or supplementing typical Western diets with high amounts of cereal grains and legumes reduces LDL cholesterol by 23% and 29% respectively [479]. Whole grains also provide the soluble fibre associated with lowered cholesterol levels and BP [469, 480, 481] and are significant sources of tocopherols, including vitamin E [482], which has been suggested as an inhibitor of LDL oxidation and oxidative cell damage [483] .

Other protection mechanisms suggested by high intake of whole grains are reductions in insulin demand and a lower body-mass index (BMI). Consuming more than a single daily serving of whole grain increases insulin sensitivity and reduces BMI, while increasing whole grain intake can increase HDL and reduce DBP [484]. A randomised, controlled clinical trial showed that, in CAD patients, consuming whole grain and legume powder reduced insulin demand, lipid peroxidation and plasma homocysteine levels [484]. Whole grain products are generally digested and absorbed slowly, because of their high viscous fibre content and physical structure [485]. This

means that whole grain has a relatively low glycaemic index which is regarded as positively correlated with reduced CVD risk. It should be noted, though, that, while whole grain's health benefits have been linked to factors such as fibre, folate and vitamin content, adjusting the results of observational studies (see Epidemiological Studies, above) for these factors shows that they are not responsible for all of the protection. Researchers, therefore, believe that whole grain's beneficial CVD effects may be mediated at least in part by other whole grain components, including polyphenols.

Human intervention studies have shown improved vascular response and endothelial function associated with a daily consumption of whole grain oat cereal, or whole grain in general [486]. A study of the effects of acute and chronic consumption of oat and wheat cereal on vascular/endothelial function following a high-fat meal showed hyperaemic blood flow was significantly decreased by acute wheat cereal and increased by oat cereal or vitamin E (though to an insignificant degree) [487].

The designs of the other human studies focused on whole grain and endothelial function have limited their contributions to our understanding in this field. One study [487], for example, used wheat cereal as a control, as opposed to a non-contributory treatment and it is probable that wheat produces effects similar to those of oats. A placebo with no known benefits (perhaps a refined rice or wheat product) might have led to more informative results. The unsuitability of the control became apparent in the results showing every treatment appearing to give sustained beneficial effects on vascular response and no significant decline during the one month of the trial, irrespective of control or oat intervention. Supplementing a diet can be problematic, for example though volunteer inconsistency. Subjects who know their diet is being observed may eat more healthily during the study, with consequent increases in vascular responses. A partial solution is a diet diary, but studies do not always report whether one was kept, or whether it was used when analysing results. Another study by the same group found that oats favourably affected vascular response in overweight subjects with dyslipidaemia to a greater extent than antioxidant vitamins E and C, after a high-fat meal, the results were not, however, significant [487].

Ferulic acid which occurs in the grain aleurone of wheat (*Triticum* spp., *Poaceae*) and other plant sources, as well as oats, has been shown to exert significant beneficial health effects, including antioxidant properties, in plasma studies. Purple wheat varieties, compared to white and red, have the highest total phenolic content and antioxidant activity, with bound ferulic, vanillic and caffeic acid levels notably abundant [488].

There is little evidence from human trials for the effect of just PAs or PAs and Av-B-rich foods in whole grain on vascular function and related mechanisms of action. Whether polyphenols/PAs in whole grain wheat can beneficially affect endothelial function is therefore insufficiently researched.

1.6.3 Investigations into Underlying Mechanisms of Whole Grain Phenolics on Vascular Function

NO, animal and *ex vivo* studies of whole grain effects on vascular function include thoracic aortic ring experiments, which suggested that FA can produce endothelium-dependent vasodilation by way of increased NO bioavailability in SHR but not normotensive WKY [489]. Further, studies have also demonstrated that ferulic acid could attenuate adhesion molecule expression in gamma-radiated HUVEC cells, and also inhibit vascular smooth muscle cell proliferation induced by Ang II [490]. L-NAME inhibited the relaxation, which would suggest that vasodilation is mediated by NO, though exposure to FA did not increase levels of cGMP which would suggest that the NO level was not enough to stimulate soluble guanylate cyclase. These findings imply a direct effect for FA on smooth muscle walls and not endothelial cells, and this needs to be further investigated.

To date there is no substantial evidence for the effects of whole grain on the RAAS**.**

1.7 Conclusion, Hypothesis and Study Objectives

1.7.1 Conclusions

In conclusion, both clinical and laboratory studies have provided wide-ranging evidence for the beneficial vascular system actions of polyphenols, including phenolic acids. While several studies have investigated potential nitric oxide-mediated mechanisms of action, few have considered the influence of phenolic acid interactions with the renin-angiotensin-aldosterone system as a potential underlying mechanism. Indeed, most interventions with cereals, including oats to date have focussed either on their antioxidant and anti-inflammatory properties, or has focussed on the action of fibre and cholesterol. As such, more trials aimed at understanding the impact of the phenolics in whole grain oats on the human vascular system are warranted, to understand better the contribution these bioactive components of oats play in defining the cardiovascular benefits of whole grain intake.

1.7.2 Hypothesis and Objectives

Overall Hypothesis: consumption of phenolic acid-rich oats will improve markers of human vascular system health (vascular blood flow and BP) through the influence of absorbed small phenolic acids on the RAAS system.

The hypothesis will be investigated using established, gold standard, clinical measures of vascular function in two randomised, controlled, human intervention trials. The oat interventions will be based on current dietary guidelines recommending that individuals consume at least one-half of all their grains as whole grains (i.e. 3 servings/day; 1 serving = 16 grams) and, therefore, represent both realistic and physiologically appropriate levels of consumption. *In vitro* experiments will be designed to show the mechanisms by which small phenolic acids from oats positively influence the RAAS, Renin expressions in juxtaglomerular kidney cells and ACE activity and levels in primary human endothelial cells (Objective 1). Their effects on the vascular system will be quantified using clinically relevant measures of vascular function, notably FMD and BP, along with a host of biochemical markers (including blood lipids, RAAS levels and activities). Urinary mineral excretions will also be assessed to shed light on the impact of oat consumption on these BP related markers (Objectives 2 and 3). Collectively, this multi-platform approach will enable the most comprehensive study to date regarding the beneficial vascular effects of oat phenolic acid consumption and will lead to a better understanding of the beneficial actions and effects and public health potential of this cheap and sustainable temperate region crop.

Overview: recent data has suggested that foods/beverages rich in phenolic acids may, as flavonoid-rich foods are known to do, positively influence human vascular function. Evidence that the favourable effects of some flavonoid-rich foods (e.g. oats) are mediated by small phenolic metabolites produced during absorption and metabolism increases the likelihood that phenolic acids have a role. At present, the best evidence for cardiovascular benefits from polyphenols comes from studies involving flavanol-rich foods, in particular cocoa and tea, however, many such studies intervene with above physiologically appropriate dosages. Flavanol studies do, however, suggest that both their acute and regular consumption result in positive cardiovascular outcomes, mainly through their effects on endothelial function, nitric oxide bioavailability and hence BP regulation. The RAAS plays a crucial role in BP homeostasis via the production of a vasoconstrictor, angiotensin II, which has a role in regulating blood volume and vasodilation independently of the NO system. The very scant body of evidence in this area points to the possibility of a role for plant polyphenols in reducing angiotensin II production, perhaps through interference with ACE and renin activities. The experimental trials in this study seek to extend this narrow background of reliable evidence, focusing on oats, which uniquely contain high levels of phenolic amides known as avenanthramides as well as small phenolic acids including ferulic acid consumed at physiologically realistic dosages. There is very limited evidence for their positive effects on the cardiovascular system, in particular BP and lipids, but we predict that due

to their structural similarity to flavanols they may be confirmed to demonstrate improvements in cardiovascular risk markers through effects on both the NO and RAAS.

The following Objectives are designed to test this hypothesis.

Objective 1: *to investigate the effects* of a range of oat phenolic compounds and their metabolites at physiologically appropriate concentrations on key enzymatic and transcription factor components of the NO and RAAS systems.

Rationale**:** polyphenols are widely reported to modulate physiological processes which leads to improved health outcomes, in particular cardiovascular health, but the mechanisms by which they exact their beneficial actions remain unclear. The two physiological processes likely involved in regulation of BP and vascular function are the modulation of NO bioavailability through interference with the eNOS pathway and interference with the RAAS, but our knowledge of both is inadequate. Phenolic compounds including some present in oats have been identified as ACE inhibitors *in vitro* in HUVEC cells, in part through interactions with amino acids at the enzyme's active site. Hence, to meet our objective, we used cultured cell lines in which these processes occur *in vitro* to: first, determine the strength of inhibition of key enzymes (renin and ACE) by a range of common polyphenols, particularly those found in oats; and second to consider in detail the impacts of the polyphenols on a range of key bioactive components of both processes. We measured renin expressions, both shed and membrane bound ACE levels and activity and compared the bioavailability of NO and NOx, in comparable trials.

Objective 2: To investigate the impact of acute phenolic acid-rich oat intake on blood pressure and vascular blood flow in healthy men with pre- or stage 1 hypertension.

Rationale: there is strong evidence for improvements in cardiovascular function including lowering of BP following acute consumption of polyphenols. Oats are a rich source of polyphenols, notably avenanthramides and phenolic acids including ferulic acid. We predict, based on whole grain and results from trials of other polyphenols, that consumption of oats will lead to improvements in vascular function through their effects on endothelial function, nitric oxide bioavailability and the inhibition of the RAAS enzymic pathways. To test this, we designed a randomised single-centre, two-arm, single-blinded, placebo-controlled crossover trial with stage 1 and pre-hypertensive volunteers, to assess the impact of a single moderate intake of oats. After consumption we measured and analysed the results of a range of previously well proven cardiovascular risk markers, notably: anthropometric, particularly BP;

microvascular blood flow, particularly FMD of the brachial artery and endothelial vasodilatory responses; blood biochemistry, particularly enzyme-linked immunoassay to investigate ACE and renin inhibition.

Objective 3: To investigate the impact of 4 weeks of phenolic acid/Avenanthramide rich oat intake on blood pressure, vascular blood flow and a range of vascular and lipid biomarkers of CVD risk in adults with pre- and stage 1 hypertension.

Rationale: while evidence has accumulated for the efficacy of plant polyphenols in acute reduction of BP, to be clinically relevant sustained improvements over months or years have to be demonstrated conclusively in chronic trials. The strongest evidence for long-term vascular improvements comes from studies of cocoa, however, cocoa and chocolate are relatively expensive-to-buy products and, therefore, not routine staples of daily diets. Likewise, many other frequently tested plant polyphenol sources. Furthermore, much of the seemingly efficacious evidence is from *in vitro* or very high dosages experiments. Our study of oats aims to determine if a relatively cheap, readily-available, sustainable foodstuff can be as effective at lowering BP at realistic dosages. We predict that sustained consumption of oats may lead to improved long-term vascular function, including nitric oxide bioavailability, inhibition of the RAAS enzymic pathways and reduced blood cholesterol. To test this, we used a trial design and subject selection similar to those of our acute trial, to assess the impact of moderate intake of oats over a 28 day period. We used two levels of Avenanthramide and phenolic acid interventions and an energy balanced but low polyphenol control. Fibre intake was standardised in all three interventions. We measured and analysed the same cardiovascular risk markers, as in our acute trial, with the addition of 24 h ambulatory BP and urinary mineral excretions.

Chapter: 2 *In vitro* Examination of the Impact of Polyphenols and their Circulating Metabolites on the Renin-Angiotensin-Aldosterone System (RAAS).

2.1 INTRODUCTION

Foods naturally high in phytochemicals, or which have been enhanced by their addition, have been shown to modulate physiological processes resulting in improved health outcomes, in particular cardiovascular health [43, 47, 48, 491-493]. It has been reported that around 80 g of specific fruits and vegetables, or beverage extracts derived from them, may contain up to 300 mg of polyphenols [57]. Polyphenols can also be found in cereals, legumes, and pulses, whose intake has long been associated with protection against cancers, diabetes and several disorders associated with poor lifestyle and aging, as well as cardiovascular diseases [53, 405, 406, 494, 495]. Notably, clinical trials have indicated that the intake of cocoa or berries leads to reductions in measurable levels of cardiovascular risk factors, such as blood pressure and enhancement of peripheral vascular function [49, 264, 309, 334, 496-498]. However, despite these promising physiological activities, the mechanisms by which they express their beneficial actions remain unclear.

Various suggestions have been made to account for this physiological activity, although the two most commonly supported by data are 1) the modulation of nitric oxide (NO) bioavailability through regulation of the endothelial nitric oxide synthase (eNOS) pathway, and 2) the regulation of the renin angiotensin aldosterone system (RAAS); the former has been more extensively investigated [46, 254, 297, 406, 499]. Evidence suggests that NO breakdown might be prevented by polyphenols or their circulating metabolites through the inhibition of enzymes that generate reactive oxygen species (ROS), including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase and lipoxygenase [375, 378]. NO production is also increased by quercetin and (-)-epicatechin in *in vitro* tests on human endothelial aortic cells and human umbilical vein endothelial cells (HUVEC) which shows that (-)-epicatechin works as an O_2^- scavenger. Quercetin was shown to cause phosphorylation of eNOS via the adenosine monophosphate-activated protein kinase (AMPK) pathway [383, 500]. There is also evidence that flavonoids increase eNOS gene expression, inducing production of prostacyclin in endothelial cells and inhibiting endothelin-1 and endothelial NADPH oxidase [398-402] In human trials, NO bioavailability has also been shown to be maintained for longer following intake of hydroxycinnamates and phenolic acids in champagne, as well as acutely improving vascular

function in fifteen healthy adults [348]. Similarly, vanillic and homovanillic acids, small phenolic acid metabolites of blueberry flavonoids, also led to a reduction in NADPH oxidase activity in neutrophils when consumed in an acute study of healthy men [349].

Many pharmacological treatments for hypertensive, cardiovascular and renal disorders have been targeted towards inhibition of the RAAS. RAAS is a multi-enzyme facilitated, hormonal system that regulates the concentration of plasma sodium, and powerfully affects arterial blood pressure [130, 131, 501, 502]. The RAAS process functions in a cascade, leading ultimately to the generation of Ang II, which is a potent vasoconstrictor, causing the narrowing of blood vessels and a rise in BP, as well as stimulating the secretion of aldosterone from the adrenal cortex [134, 136, 138, 139]. Ang II acts on various organs and tissues throughout the body, including the kidneys, where it decreases the glomerular filtration rate [131]. Ang II also increases cardiac output and stimulates the hypothalamus to release antidiuretic hormone (ADH) (arginine vasopressin), from the posterior pituitary gland which leads to greater reabsorption of water by the kidneys [140] which results in an increase in extracellular fluid volume within the body, and so increased BP [140, 142, 143]. Suppression of renin and angiotensin converting enzyme (ACE), two of the principle RAAS hormones, are two of the strategies used for reducing blood pressure, maintaining electrolyte homeostasis, and regulating haemodynamics and volume status [503-505], although adverse effects on the kidneys are frequently encountered with such pharmacological treatments. As such, there is an interest in finding novel RAAS inhibitors without such unwanted side effect and among those being investigated are polyphenols [253, 506].

Modulation of the RAAS may occur through inhibition of ACE, which would lead to increased NO production [507], as has been demonstrated by studies using pomegranate juice, rooibos tea, green tea and polyphenols (especially flavonoids), which found that ACE activity was inhibited and angiotensin II (Ang II) is not formed. Vasoconstriction does not, therefore, take place and consequentially blood flow is maintained or increased [350, 351]. For example, intake of pomegranate juice polyphenols by hypertensive patients led to a 36% reduction in serum ACE activity [254] and caffeic acid and its derivatives may be capable of modulation of the RAAS, acting by reducing renin, ACE and aldosterone production [361]. Garden egg fruit, with enzymes α-amylase, α-glucosidase, demonstrated strong inhibition of ACE activity in rat lung homogenates *in vitro* [370]. In *in vivo* studies of humans, food components like pomegranate juice, rooibos tea, green tea and polyphenols (especially flavonoids) have been shown to have inhibit ACE activity, hence Ang II is not formed and its vasoconstrictive function is lost and blood volume is increased [350, 351].

Another target for blood pressure reduction within RAAS in renin, which is primarily expressed in myoepithelioid granulated cells of the kidney, called juxtaglomerular cells, situated in the afferent arteriole. Within juxtaglomerular cells, cyclic adenosine monophosphate (cAMP) is thought to play a major controlling role in renin production and release, with the transcription factors cAMP-response element-binding protein-1 (CREB) and cyclic AMP-dependent transcription Factor (ATF-1), and subsequent activation of protein kinase A (PKA), also necessary to activate extracellular signal-related kinase (ERK) pathway [508-512], angiotensin induced activation of ERK1/2 is NADPH oxidase independent but is cAMP/PKA-dependent in mesangial cells. Following renin expression in the cell it cleaves the N-terminus of angiotensinogen to initiate an enzymatic cascade by producing angiotensin I (Ang I) which is released into circulation and further cleaved by ACE to produce Ang II, a vasoconstrictor [513, 514]. Inhibiting renin therefore blocks the RAAS pathway through prevention of Ang II production, Ang II production leads to vasoconstriction and an increase in blood pressure (BP). In As4.1 cell, CREB-1 and cyclic AMP-response element modulator (CREM) bind to the cAMP-responsive element (CRE) consensus binding site [515] and inhibit renin expression.

There are relatively few studies relating to the influence of polyphenols and their metabolites on enzymatic inhibition of the RAAS especially renin, hence their mechanisms of action on the RAAS are poorly understood [366, 516-518]. However, studies on the modulation of ACE activity have generally incorporated polyphenol interventions at high concentrations, so their physiological relevance is limited. To generate more informative results regarding renin inhibition and the physiological effects of its release from cells, interventions comprising physiologically realistic plasma concentration post intakes have been used in this study.

Since most of the evidence for the protective characteristics of polyphenols against stress relevant to chronic disease pathophysiology is derived from *in vitro* studies, juxtaglomerular cells have frequently been used as a model to study renin inhibition and the impact of polyphenols on renin [519-521]. Similarly, vascular endothelial cells from human umbilical veins (HUVEC) are a standard model used to study endothelial-derived vasoactivity, such as endothelial NO synthase and prostacyclin. Therefore, these cells provide a suitable model in which to study changes in endothelial NO, ACE levels and vascular function [522-525]. In this study we use an *in vitro* model to investigate the effects of several polyphenols found in oats with suitable comparator polyphenols found in many of the polyphenol-containing foods/beverages that have been shown to induce physiological improvements in vascular function. In particular we consider the effects of oat phenolic compounds and their metabolites on RAAS using juxtaglomerular and HUVEC cells. We specifically assess whether polyphenols and their circulating metabolites influence renin

expression and upstream kinases and transcription factors which control renin release in juxtaglomerular cells. Furthermore, we assess whether oat phenolics affect ACE activity and levels in HUVEC, to better understand the mechanisms of polyphenol actions. The results from these *in vitro* studies provide evidence that the RAAS is an important mechanism in the body by which polyphenols produce some of their cardiovascular benefits.

2.2 MATERIALS AND METHODS

2.2.1 LIST OF POLYPHENOLS USED IN THE EXPERIMENTS.

(-) Epicatechin (EC), avenanthramide-B (Avn-B), avenanthramide-C (Avn-C), trans-ferulic acid (t-FA), 2, 4-dihydroxybenzoic acid (2,4-DHBA), vanillic acid (VA), 3-(4-hydroxyphenyl) propionic acid (3,4-OHPPr), 4-hydroxybenzoic acid (4HBA), caffeic acid (CA), sinapic acid (SA), ferulic acid-glucuronide (FAG) and isoferulic acid-sulphate (IFAS) were dissolved in methanol (34860) obtained from Sigma-Aldrich (Gillingham, UK).

2.2.2 OTHER MATERIALS

In vitro Toxicology Assay, Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (M-5655), MTT Solubilisation solution (M-8910) (Sigma, UK), Genios Microplate Reader (Genios Microplate Reader, TECAN Group Ltd., Switzerland). Renin-expressing cell line (As4.1 cells) (CRL-2193), Dulbecco's Modified Eagle's Medium (30-2002), (LGC Standards, UK).

Penicillin-Streptomycin (15070-063), 7-TrypLE™ Select Enzyme, NO phenol red (12563-011) (Life Technologies Ltd, USA). PBS (14190086) was purchased from (Thermo Fisher Scientific Inc., Roskilde, Denmark). RNeasy Mini Kit (74104), RNase-Free DNase Set (79254), RT2 First Strand Kit, Polymerase Chain Reaction: (330401), QuantiTect SYBR Green PCR Kit (204141), RNase inhibitor(129916) mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer (Mm_Gapdh_3_SG, QT01658692) (Qiagen, Netherlands), 7300 Real-time PCR system (AB Applied Biosystems, California, Unite States), Primer Renin F Sequence TGA-AGA-AGG-CTG-TGC-GGT-AGT, Primer Renin R Sequence TCC-CAG-GTC-AAA-GGA-AAT-GTC, RNaseZap® RNase Decontamination Solution (Life Technologies Ltd, USA) and Tris-EDTA buffer solution (Sigma-Aldrich, UK). Pierce™ BCA Protein Assay Kit (23227), RIPA Buffer (89900), Halt™ Protease and Phosphatase Inhibitor Cocktail (78440), iBlot® Transfer Stacks, Nitrocellulose (IB23002), ECL prime western blotting det (10308449) (Thermo Fisher Scientific, USA), Precision Plus Protein™ WesternC™ Blotting Standards (1610385), 10 % Mini-PROTEAN® TGX™ Precast Protein Gels, 50 µl well (IB23002) (Thermo Fisher Scientific, USA), 2x Laemmli Sample Buffer, 2-Mercaptoethanol (Bio-Rad, USA), Marvel Original Dried Skimmed Milk (Iceland), Sodium chloride (S7653), Trizma® hydrochloride

(T3253), Bovine Serum Albumin (A7906), Trizma® base Primary Standard and Buffer (T1503), Glycine (G8898), Sodium dodecyl sulphate (L4509) TWEEN® 20 (P1379) from (Merck, Germany), Anti-rabbit IgG, HRP-linked Antibody, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (4370), p44/42 MAPK (Erk1/2) (L34F12) Mouse mAb (4696), Phospho-CREB (Ser133) (1B6) Mouse mAb, CREB (86B10) Mouse mAb, Anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology, USA). Coupled antibody pair (Duo Set Kit, DY929 R&D, Wiesbaden, Germany), Colour Reagent A (H2O2) and Colour Reagent B, Tetramethylbenzidine, Capture Antibody, ACE (CD143) specific mouse monoclonal antibody, Angiotensin Converting Enzyme from rabbit lung (A6778), FAPGG (F7131), CHAPS 100 mM solution(19899), N-Hippuryl-His-Leu hydrate (H1635), Phthaldialdehyde (79760), 5-Hydrochloric acid, Glycerol, Captopril powder (Sigma-Aldrich, Germany), Human Umbilical Vein Endothelial Cells, Pooled (HUVEC) (C2519A), EGM-2 (CC-3162), Cryo amp EGM-2 Bullet Kit (CC-3156 & CC-4176) (LONZA,UK), Nitrate/Nitrite Colorimetric Assay Kit (CAY780001) (Cambridge Bioscience Ltd, UK), Human ACE Quantikine ELISA Kit (DACE00), Quantikine Immunoassay Control Set 839 for Human ACE/CD143 (QC32) (R&D Bio-Techne Ltd, UK), Angiotensin Converting Enzyme from rabbit lung(ACE) (A6778).

2.2.3 CELL CULTURE AND GROWTH

2.2.3.1 AS4.1 CELLS

As4.1 cells are a renin-expressing clonal cell line derived from the kidney neoplasm of a transgenic mouse [520]. Cell were cultured at 37 °C in a humidified atmosphere with 95 % oxygen and 5 % CO_2 in DMEM medium supplemented with 10 % FBS and 1 % Penicillin-streptomycin [526, 527]. The medium was changed every two days to increase growth rate. After conspicuous confluence, the cells were washed with a solution of PBS free from calcium and magnesium ions, followed by trypsinization, creating a single-cell suspension. The resultant cell suspension was centrifuged at 100 x g for 5 min at 20 ˚C (Centrifuge C 12, AWEL Industries, France). The supernatant was then removed by suction. Fresh culture medium was added to create a new single-cell suspension with the desired seeding cell number quantified per 1 mL volume. Seeding was done using a 75m^2 tissue culture flask. With a haemocytometer and trypan blue exclusion, cell counting was carried to determine cell number. Cell suspensions with the desired seeding number were distributed evenly onto several flasks.

2.2.3.2 HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Pooled human umbilical vein endothelial cells (HUVECs) (TCS CellWorks, Buckingham, UK) were used between passages 2 to 4 [263, 528, 529]. Cells were routinely cultured in Nunclon™ Δ 75cm^2 flasks in endothelial cell growth medium (TCS CellWorks; proprietary basal medium formulation

supplemented with growth factor, antibiotics gentamicin & amphotericin B and 2 % v/v foetal bovine serum) at 37°C and 5 % CO_2. HUVECs were sub-cultured using 0.025 % trypsin and 0.01 % EDTA (TCS CellWorks).

The cells were sub-cultured at 70-85 % confluence. For each 25 cm^2 of cells to be sub-cultured, 5 ml of the medium from one culture vessel was aspirated and the cells rinsed at room temperature. Subsequent to the aspiration from the flasks, the cells were ensconced with 2 mL of Trypsin/EDTA solution and the culture vessels placed into a 37°C humidified incubator for 3-5 minutes. Microscopic examination of the cell layer was done periodically to check for cell separation. The process of trypsinization was allowed to continue until approximately 90 % of the cells are rounded up. After cell release, the trypsin was neutralized in the flask with 5 mL of Trypsin Neutralizing Solution at room temperature and the detached or separated cells quickly transferred to a sterile 15 mL centrifuge tube. The flask was rinsed with a final 2 mL of HUVECs to collect residual cells, and the rinse added to the centrifuge tube. The thus harvested cells were then centrifuged at 200 x g for 5 min to collect pellet of the cultured cells after aspiration of most of the supernatant.

2.2.4 PREPARATION OF POLYPHENOLS

Epicatechin, avenanthramide-B, avenanthramide-C, trans-ferulic acid, 2,4-dihydroxybenzoic acid, vanillic acid, 3-(4-hydroxyphenyl) propionic acid, 4-hydroxybenzoic acid, caffeic acid, sinapic acid, ferulic acid-glucuronide and isoferulic acid-sulphate, were each dissolved in 70 % methanol as 3 mg/ml stock. The final concentration of solvent in experiments was ≤ 0.5 %. All stocks were kept at −80°C.

2.2.5 EVALUATION OF AS4.1 CELLS VIABILITY

Cytotoxicity of selected polyphenols is determined by an Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay as described previously in [530, 531], which utilizes the outcome based on mitochondrial conversion of the tetrazolium salt (3- [4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltertrazolium bromide). In this study, an MTT assay has been employed to assess the quantity of viable AS4.1 juxtaglomerular (JG) cells following a 24h polyphenol exposure in various physiological relevant concentrations (i.e. 0.1 - 50 µM). Cells were plated in a 24-well culture plates (Thermo Fisher Scientific Inc., Roskilde, Denmark) calibrated at 5×10^4 cells per well in 500 µL of media followed by 24 h adherence at 37˚C with 5 % CO_2. On reaching the confluence, the medium was replenished with fresh medium containing various doses of polyphenols (100 nM, 500 nM, 1.0 µM, 5 µM, 10 µM and 50 µM) in triplicate and again incubated for 24 h at 37 ˚C. Each well in the 24-well plate was then administered 20 µL of MTT solution (5 mg/ml in PBS, M-5655,

Sigma), followed by incubation at 37˚C for 4 h. After incubation, the MTT solution in the medium was extracted by pipetting. In order to solubilise the formazan crystals formed, 100 µL of solubilisation solution (M-8910, Sigma) were added to each well, and the plates mildly shaken for 20 Seconds. For each sample, absorption of three replications were measured at 595 nm with a *Genios Microplate Reader* (*TECAN Group Ltd., Switzerland*) and their mean value was used for the final result. Absorbency values were plotted against counted cell numbers in order to establish a standard calibration curve. MTT absorbency was used to determine viable cell numbers on meshes or culture-well bottom from the standard curve; this procedure was replicated twice.

2.2.6 RENIN INHIBITION ASSAY

The human recombinant renin inhibitor screening assay kit *(CAY10006270-96 wells) (Cambridge Bioscience Ltd, UK)* comprising human recombinant renin (*1006270)*, *S*ubstrate (Arg- Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dabcyl)- Arg) and Tris-HCl buffer (50 mM, pH 8.0, containing 100 mM NaCl) (*Cayman Chemical Co. Ann Arbor, MI, U.S*.). The procedure was carried as per the manufacturer's instructions and described previously [532-535]. Prior to each assay, renin enzyme was freshly prepared by diluting it 1:20 in Assay buffer (50 mM Tris-HCl, pH 8.0, and 100 mM NaCl). A 95 µM solution of renin substrate (Arg-Glu(EDANS)-Ile-His-Pro-Phe-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-Arg) in dimethyl sulfoxide (DMSO) was ready to use as supplied (10006872, *Cayman Chemical Co*). For each assay, background wells were used by adding 20 µL of substrate, 160 µL of Assay buffer and 10 µL of solvent (methanol). The activity wells (control) were prepared by adding 20 µL of substrate, 150 µL of Assay buffer and 10 µL of solvent, and the inhibitor wells were prepared in the same way but with 10 µL of inhibitor (polyphenols) instead of solvent. All reactions were tested in triplicate. The reaction was initiated by the addition of 10 µL of renin to activity and inhibitor wells. Plates were gently mixed for 10 seconds and left to incubate at 37 ˚C for 15 minutes. Plates were measured using a Genios microplate reader (TECAN Group Ltd., Mannedorf, Switzerland) using excitation wavelengths of 340 nm and emission wavelengths of 465 nm.

The renin inhibitory activity was calculated as percentage of inhibitory activity (Inhibition %) with the following equation.

$$\%\, Inhibition \;=\; \left[\frac{AF\ (Control) - AF\ (Sample)}{AF\ (Control)}\right] \times 100$$

The analysis was performed based on the dose-dependent suppression of renin activity with three different concentrations of the inhibitors in triplicate, and then determined the average fluorescence. The IC_{50} value, defined as the concentration of the inhibitor required to inhibit 50 % of the renin activity, was calculated using the linear function of percentage renin inhibition versus the logarithm of the inhibitor concentration in accordance to previous published paper [499].

2.2.7 RENIN GENE EXPRESSION BY RT-PCR

2.2.7.1 STIMULATION OF CELLS WITH DIFFERENT CONCENTRATIONS OF POLYPHENOLS

The cultured As4.1 JG cells (described in Section 2.2.3.1) were seeded at a density of 1×10^6 in a 25 cm^2 tissue culture dish. Upon reaching confluence (approximately 7×10^6 cells), the individual polyphenols prepared as described in (Section 2.2.4) were added in carrier medium at different concentrations (100 nM, 500 nM and 1.0 µM) in order to stimulate the cells. Cells were then incubated at 37 ˚C with 5 % CO_2 for 20 h.

2.2.7.2 EXTRACTION OF RNA AND CDNA SYNTHESIS

After stimulation, the RNeasy Mini Kit was used according to manufacturer's instructions to extract the cellular RNA. RNA concentrations and quality were determined using NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Delaware, US). After extraction, RNA samples were treated with the RNase-Free DNase Set to further minimise presence of DNA. The samples were then stored at -80° C until further use.

For synthesis of complementary DNA (cDNA), 1 µg of RNA and the RT2 First Strand Kit were used adhering to manufacturer's instructions. Purified DNA was stored at -20° C until gene expression was measured.

2.2.7.3 RENIN GENE EXPRESSION MEASUREMENT

Renin Gene Expression was measured as described previously [536].The mouse Mm_Gapdh_3_SG primer was used as internal control gene (housekeeping gene), Renin R and Renin F primers were used (Table 2.1). Each primer was supplied dry and reconstituted in 1.1 ml TE buffer [1M Tris pH 8.0 and 0.05 M EDTA in RNase-free water] and then were stored at -20^o C.

Primer I.D.	Sequence
Mouse Renin F	5' - TGA-AGA-AGG-CTG-TGC-GGT-AGT
Mouse Renin R	3' - TCC-CAG-GTC-AAA-GGA-AAT-GTC

Table 2.1 Primer used for RT- PCR

For amplification, the QuantiTect SYBR Green PCR Kit was used and following reaction mix was prepared by mixing: 2.5 µL of cDNA, 12.5 µL of SYBR green master mix, 2.5 µL of each primer and 7.5 µL of RNA-free water. Subsequently, the 96-well plate was vortexed and spun to remove bubbles using Heraeus Multifuge 3SR Plus (Thermo Scientific, UK). Amplification was carried in a Thermal cycler (7300 Real-time PCR system, AB Applied Biosystems, California, United States) with the following settings; an initial denaturation for 15 min at 95°C, followed by 40 cycles of 15s at 94°C and 30s at 55°C. The final step comprised of 30s at 72°C. Following amplification, melting curve analysis was performed to verify the specificity of the reactions.

The relative levels of gene expression were determined from the real-time PCR efficiencies using the Delta Delta Ct method (the $2^{-\Delta\Delta CT}$) [537]. Results are presented relative to the control cells grown without polyphenols (which is arbitrarily defined as 1). The expression of the target gene was normalised to GAPDH expression (housekeeping gene). Statistically significant differences were determined using a GLM then post hoc analysis (Tukey adjustment) in SPSS.

2.2.8 WESTERN IMMUNOBLOTTING

Western immunoblotting was undertaken to study the underlying mechanisms of gene expression in PCR outcomes with reference to proteins CREB, ATF-1 and ERK1 and 2. Western immunoblotting described in [538].

2.2.8.1 LYSATE PREPARATION

Cells were grown as described in Section 2.2.3.1, in 25 cm^2 flask at a seeding concentration of 2×10^6 cells, at 37 °C with 5 % CO_2 in DMEM medium. After conspicuous confluence, the cells were washed with a solution of PBS free from calcium and magnesium ions, followed by trypsinization, cells were exposed for 1 h to media containing different polyphenols, each at a concentration of 1 µM. Media was then removed and cells washed with PBS followed by addition of 250 µL of RIPA buffer, 2.5 µL of Protease and Phosphatase Inhibitor. Lysates tubes were mixed and incubated on ice for 20 min, passed through needles 19G–25G, vortexed again and left on ice 5 min. Finally, the lysates were pelleted by centrifugation at 1400 ×g for 12 min at 4 °C. The cell pellets were stored at -20 °C until western blot analysis. Protein content was determined by the *BCA Protein Assay Kit (Pierce, USA)* and quantified with *a Genios Microplate Reader (TECAN Group Ltd., Mannedorf,*

Switzerland) at a wavelength of 540 nm. The concentrations of the cell lysate were then calculated from the standard curve of the BCA samples of known concentration.

2.2.8.2 WESTERN BLOT: PHASE I (PROTEIN SEPARATION)

Gradient 10 % pre-casted gel (stain-free) (Bio-Rad, Hertfordshire, UK) was used to separate the protein samples according to their molecular weight. Proteins mixed with equal volume of 2x Lamaelli buffer supplemented with 2-mercaptoethanol (50 µl in 950 µl) (Bio-Rad) then heated at 100 °C for 3 min in a water bath, heated to lyse cells and denature proteins. 5 µl of Precision plus protein standard (ladder) in the range of 10-250 kDa (Bio-Rad) were loaded as a marker in each gel stain-free ladder. And 20 µg of protein samples were loaded into the wells. The main gel tank was filled with 1x running buffer [25 mM Tris pH 8.3, 0.1 % (w/v) SDS, 192 mM glycine]. The gel was run at 200V for about 25 minutes, until the blue dye was at the bottom of the gel.

2.2.8.3 WESTERN BLOT: PHASE II (TRANSFER)

To transfer the gel to the membrane, a nitrocellulose membrane (iBlot® 2 Transfer Stacks) with a pore size of 0.2 µm was used. The membrane was placed into the iBlot® 2 Gel Transfer system (Thermo Fisher Scientific, USA). After ensuring no bubbles were present, and the transfer was set up at 1.3 A constant current for 7 minutes. After which the membrane was washed with 25 ml Tris buffered saline (TBS) [0.1 % Tween-20, 150 mM NaCl, 2 mM KCl and 50 mM Tris pH 7.4]. Washing steps took 5 min each and were carried at room temperature with gentle rocking. Washing was followed by blocking for 1 h with 5 % (w/v) dried milk in TBS-T 25 mL of blocking buffer at room temperature with gentle rocking. The gels were then washed again 3 times with 15 mL TBS.

2.2.8.4 PHASE III (PROTEIN DETECTION)

The membrane was incubated with appropriate primary antibody at the dilution shown in Table 2.2 diluted in 5 % w/v BSA (Sigma) in TBS-T 5 mL of primary antibody dilution buffer in a heat-sealable bag with gentle agitation for 1h and 30min at room temperature.

The membrane was then washed three times with of TBS-T for 5 min each and then incubated with appropriate HRP-linked secondary antibody and the marker conjugate (Precision Plus protein StrepTactin-HRP conjugate) at (1:5000) to detect biotinylated protein markers. Both diluted in TBS-T and 5 % w/v dried milk for 30 min with gentle agitation at room temperature. The membrane was washed thrice for 5 min on a rocking platform with TBS-T.

Antibody	Source	Dilution
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) (Primary Antibody).	Rabbit	1:2000
p44/42 MAPK (Erk1/2) (L34F12) (Primary Antibody).	Mouse	1:2000
Phospho-CREB (Ser133) (1B6) (Primary Antibody).	Mouse	1:2000
CREB (86B10) mAb (Primary Antibody).	Mouse	1:2000
Anti-mouse IgG, HRP-linked Antibody (secondary antibody).	Mouse	1:5000
Anti-rabbit IgG, HRP-linked Antibody (secondary antibody).	Rabbit	1:5000
Precision Plus protein StrepTactin-HRP conjugate		1:5000

Table 2.2 List of antibodies used for immunoblotting

The results represented the average ± SEM (n = 3) of biological replicates generated from 3 independent cell cultures and were analysed for statistical significance using GLM with post hoc analysis (Tukey adjustment).

Bands were detected using enhanced chemiluminescence (ECL) (GE Healthcare) according to the manufacturer's instructions and quantitation was done digitally using a Luminescent Image Analyser (Image Quant LAS4000mini, GE Healthcare). The Image was analysed using ImageJ software, the intensity of each band was obtained in numerical form. Results were expressed as a ratio to the relevant loading control (total protein), band signals were normalised relative to digitally quantified total protein using the Bio-Rad Stain-Free System.

2.2.9 POLYPHENOLS AND ANGIOTENSIN CONVERTING ENZYME

Evaluation of the effects of polyphenols on ACE enzyme was carried out with HUVEC cells with four experimental sequels; first shed ACE (enzyme linked immunosorbent assay (ELISA), second membrane-bound ACE ELISA measurement and third measurement of ACE activity.

2.2.9.1 SHED ACE ELISA

Shed ACE was measured in culture supernatant of HUVEC by Human ACE Quantikine ELISA Kit commercially available ELISA (Duo Set Kit, DACE00 R&D, Wiesbaden, Germany), methods described previously [539, 540]. HUVEC cells grown in 6-well plates were washed 3 times with PBS and incubated with different polyphenols (1.0 µM) diluted in 3 mL of culture medium in triplicate. After 24 hours, the supernatant of the aqueous medium was collected and centrifuged at 1,500 rpm for 10 min at 5˚C. The monoclonal antibody specific ACE was supplied attached onto microplate by the company. The desired antigen (ACE) is then added and bound to the immobilized antibody on the microplate, hence an enzyme-linked monoclonal antibody (detection antibody) is added. Furthermore, a substrate solution is added to the wells, which produces a detectable signal due to a change in colour. The samples were prepared in a 10-fold dilution adding Calibrator Diluent RD6-45 (180 µL) to tested polyphenols samples (20 µL). Using a Human

ACE Standard stock (100 ng/mL) and Calibrator Diluent RD5P, a serial dilution was carried at the following concentrations: 50, 25, 12.5, 6.25, 3.13, 1.56, 0.781 and 0 ng/mL. High, Medium and Low controls were also used.

All reagents and samples were brought to room temperature before used and all samples, standards, and controls were assayed in duplicate. All reagents, standard dilutions, and samples were prepared as directed in the product insert. To each well 100 µL of assay diluent was added followed by 50 µL of standards, controls and sample. The plate was covered with a plate sealer and incubated at room temperature for 2 h on a horizontal orbital microplate shaker (Stuart SSM5 Shaker, Microtitre, Mini, Cole-Parmer, Staffordshire, UK) at 500±50 rpm. The wells were aspirated and washed by wash buffer (20 mL to dH_2O and filled up to 500 mL) 4 times in total using a multichannel pipet (Sartorius Stedim Plastics GmbH, Germany). After every washing step, the liquid was removed completely by blotting against clean paper towels. After the last aspiration step, 200 µL of human ACE conjugate was added to each well. A new plate sealer was used to cover the plates and the wells incubated at room temperature for 2 h on the shaker. After repeating the washing procedure 4 times, 200 µL of substrate solution was added to each well. The plates were then incubated for 30 min on the bench at room temperature and protected from light. Finally, 50 µL stop solution was added to each well and the plates were measured at 450 nm within 30 min using a microplate reader was determined in Spark ® multimode microplate reader (TECAN Trading AG, Switzerland) set to 450 nm. Since wavelength correction was available, the reader was set to 570 nm to avoid subtraction during calculation.

2.2.9.2 MEMBRANE-BOUND ACE ELISA

Cell were grown and treated as described for shed ACE (2.2.9.1). For membrane-bound ACE the wells washed with 1 mL PBS followed by addition of 50 µL of RIPA buffer with 0.5 µL of Protease and Phosphatase Inhibitor in each well. Then scraped off the wells using a rubber scraper and the cells suspended in ice cold PBS, lysates tubes were mixed, then incubated on ice for 20 min, vortexed again, left on ice 5 min, and pelleted by centrifugation at 1400 ×g for 12 minutes at 4 °C. The cell pellets were stored at -20 °C until analysis. Assay procedure is as shed ACE and described previously [539, 540].

2.2.9.3 ASSAY FOR ACE ACTIVITY

ACE activity was measured in HUVEC using ACE substrate (Hip-His-Leu) as described previously by [541]. Briefly, cells were treated with different polyphenols (1.0 µM) in 6 well plates for 24 hours followed by washes with phosphate buffered saline. Cell lysis was performed with 100 µL/well of 8 mM CHAPS in PBS for 15 min at room temperature. Then, scraped off the dish using a rubber

scraper from the wells and vortexed. To substantiate the ACE activity in the sample, the activity of membrane-bound ACE was measured in a homogenate of cultured HUVEC using ACE substrate (Hip-His-Leu). Followed by addition of 15 µL of cell homogenate to 200 µL of ACE substrate (1 mM Hip-His-Leu) and incubated for 60 min at 37 °C. The reaction was terminated with 25 µL of 0.28 M NaOH and the His-Leu product was estimated by 10 min incubation with 10 µL of O-phthaldialdehyde (20 mg/mL in methanol). After addition of 20 µL 3M HCl, the fluorescence of samples was measured using 365 nm excitation/500 nm emission filters.

2.2.10 NITRITE AND NITRATE ANALYSIS

Cells were grown and treated as described for shed ACE (2.2.9.1). After 24 h, the supernatant of the aqueous medium (1 mL) was collected and centrifuged for nitrite and nitrate analysis with methods described by [542-545]. The initial step is conversion of nitrate to nitrite, then addition of the Griess Reagents which convert nitrite into a purple azo compound. Photometric measurement of the absorbance accurately determines NO_2 concentration. Total nitrate and nitrite were measured by Nitrate/Nitrite Colorimetric Assay Kit (Cambridge Bioscience Ltd, UK), 200 µL of assay buffer were added to the blank wells, 80 µL of standards or samples added to the wells in duplicate. The standards were diluted with assay buffer as following: 35, 30, 25, 20, 15, 10, 5 and 0 µM. The, 10 µL of the enzyme Cofactor mixture was added to standard and sample wells, followed by 10 µL of Reductase. The plate was then covered and incubated at room temperature for 2 hours. Incubation was followed by the addition of 50 µL of reagent R1 and 50 µL of reagent R2 in each well. The colour was allowed to develop for 10 minutes at room temperature. Total nitrite was measured at 540 nm absorbance using a TECAN micro plate reader.

For measurement of nitrite, 200 µL of assay buffer was added to blank wells, and 100 µL of standards and samples were added in duplicate to each well. This was followed by 50 µL of reagent R1 and 50 µL of reagent R2. The colour was allowed to develop for 10 minutes and then measured at 540 nm absorbance using a TECAN micro plate reader.

Total NO, nitrate and nitrate was calculated in (μM) with the following equations.

$$(Nitrate + Nitrite)(\mu M) = \left(\frac{A540 - y - intercept}{slope}\right) * ((200\mu L))/samplevolume\mu L))$$

$$Nitrate\ (\mu M) = \left(\frac{A540 - y - intercept}{slope}\right) * ((200\mu L)/samplevolume\mu L))$$

$$Nitrate(\mu M) = (Nitrate + Nitrite) - (Nitrite)$$

2.2.11 STATISTICAL ANALYSIS

At least 3 biological replicates were carried out for each assay. All statistical analyses were carried out using the statistical package for social science (SPSS, version 24; SPSS Inc., USA). The data are presented as means ± SEM. A LM was used to show significant (P ≤ 0.05) effects of independent (predictor) variables on the response variable and covariables. A similar method was used for HUVEC cell experiments, to analyse the effects of polyphenols and time after treatment as independent variables on measurements of the assayed indicators as dependent variables. Tukey's comparison (P≤ 0.05) was used for *post hoc* analysis of significant results.

2.3 RESULTS

2.3.1 CYTOTOXICITY OF POLYPHENOL COMPOUNDS ON AS 4.1 CELLS

Cytotoxicity of polyphenols was assessed in AS4.1 cells (as described in Section. 2.2.5). The dose response curves for cytotoxicity as assessed by MTT are presented in Figure 2.1. Cells were treated with different polyphenols at different concentrations (0.1 µM, 0.5 µM, 1.0 µM, 5 µM, 10 µM and 50 µM) in control media for 24 hours. The polyphenol concentrations ranging from 0.1 to 10 µM did not affect cell viability as no appreciable cytotoxicity was observed. However, a significant, sharp decline in cell viability was observed at a concentration of 50 µM, where throughout all treatments the cell viability was less than 30 % ($p < 0.001$). It was also observed that at a concentration of 10 µM of trans ferulic acid (t-FA), cell viability was reduced to 65.33 %. The maximum plasma concentration of polyphenols rarely exceeded 1 µM following interventions of 10 – 100 mg of a single phenolic compound [546]. Concentrations at 1.0 µM were therefore considered to be most appropriate for further assays.

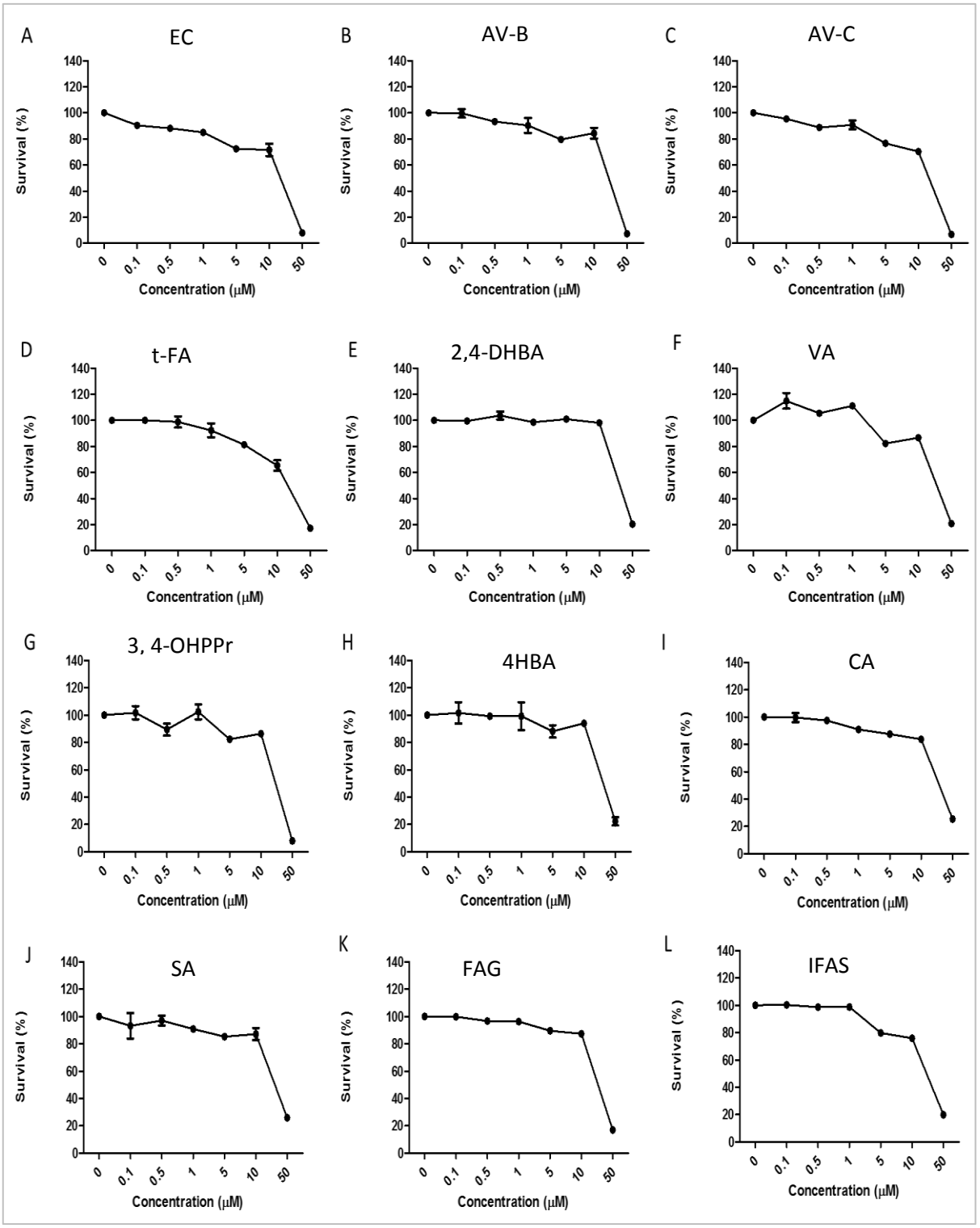


Figure 2.1 Cytotoxicity of polyphenols on As4.1 cells

Graphs of viability of As4.1 cells incubated for 24h at different concentrations (0.1, 0.5, 1, 5, 10 and 50 µM) of various polyphenols (see Section 2.2.1 for full names). Each letter corresponds to a specific compound A=EC, B=AV-B, C=AV-C, D=t-FA, E=2,4 DHBA, F=VA, G=3,4-OHPPr, H=4HBA, I=CA, J=SA, K=FAG and L=IFAS. Cell viability was measured with MTT assays. Each of the 6 concentrations was assessed in triplicate. Error bars depict the primary results based on standard error.

2.3.2 RENIN INHIBITION ASSAY

To investigate how different polyphenols could inhibit renin enzyme activity, a renin inhibitory assay was performed (Figure 2.2, Table 2.3). Obtained data indicate that tested polyphenols exert renin inhibition in a concentration dependant manner, from 0.1 - 1 µM. It was observed that t-FA possessed the strongest activity with inhibition ranging from only 6.43 at 0.1 µM to 79.81 % at 1 µM, with an IC_{50} value of 0.51 µM (Table 2.3). EC exhibited an inhibitory potency close to that of 2,4-DHBA with an IC_{50} value of 0.52 and 0.54 µM respectively and with a maximum inhibition of 86.41 and 90.48 % at 1 µM concentration. AV-B and 3,4-OHPPr showed similar inhibition levels of renin activity with 90.78 and 85.42 % with an IC_{50} of 0.57 µM for both. VA exerted renin inhibition activity ranging from only 16.33 to 71.43 % with an IC_{50} value of 0.60 µM. In contrast, AV-C, 4HBA, SA and IFAS exhibited weak inhibition, with IC_{50} values greater than 1 µM. The renin inhibitory activities of the 12 compounds are presented in Figure 2.2 and Table 2.3.

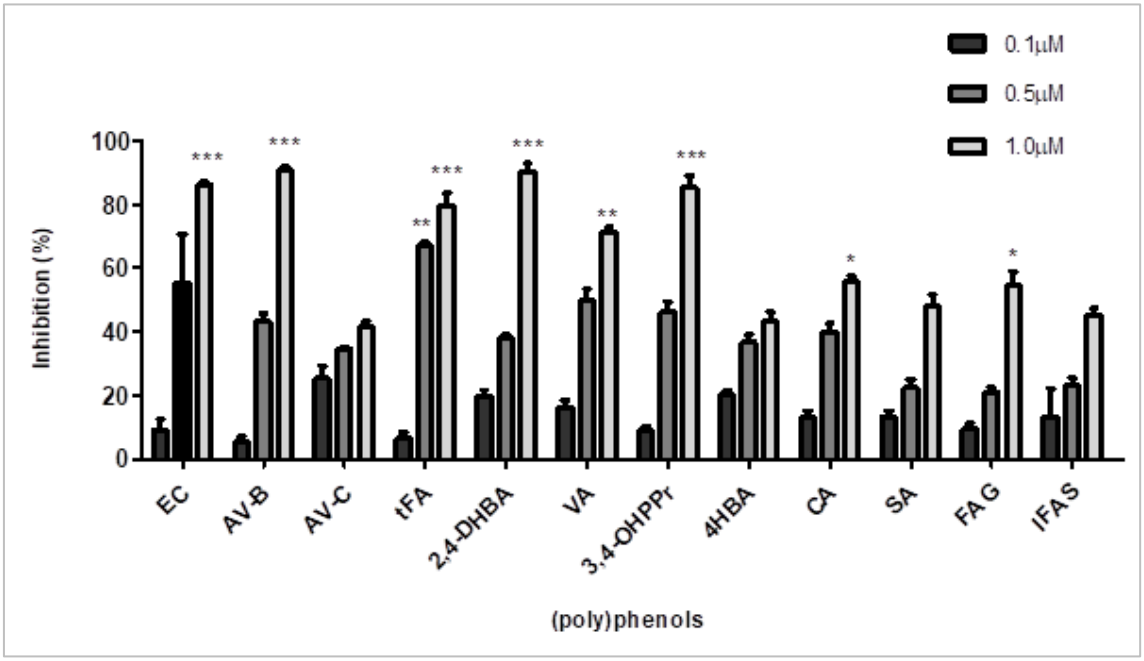


Figure 1.2 Renin inhibitory activity of selected polyphenols

Graph of inhibitory effects of selected polyphenols (see Section 2.2.1 for full names), at concentrations of 0.1,0 .5, and 1.0 µM, on Renin. Results are expressed as percentage inhibition of renin activity (means ± SEM) in triplicate.

Polyphenol compound	IC_{50} (µM)
EC	0.52
AV-B	0.57
AV-C	> 1
t-FA	0.51
2,4-DHBA	0.54
VA	0.60
3,4-OHPPr	0.57
4HBA	> 1
CA	0.82
SA	> 1
FAG	0.95
IFAS	> 1

Table 2.3 Approximation of IC_{50} values of tested polyphenols against renin activity.

The oat and comparator polyphenols used in this study with the concentrations at which IC_{50} occurred.

2.3.3 Effect of Polyphenols on Renin mRNA Levels

The effects of polyphenols on renin mRNA expression were assessed in AS4.1 cells by RT-PCR and gene expression was quantified via real-time RT-PCR with specific primers and normalised to GAPDH expression (as described in section 2.2.7). For this study, polyphenols were used at 3 different concentrations (0.1, 0.5 and 1.0 µM) and gene expression was measured after 24 hours of exposure. Overall, and when compared to controls, the tested compounds decreased renin

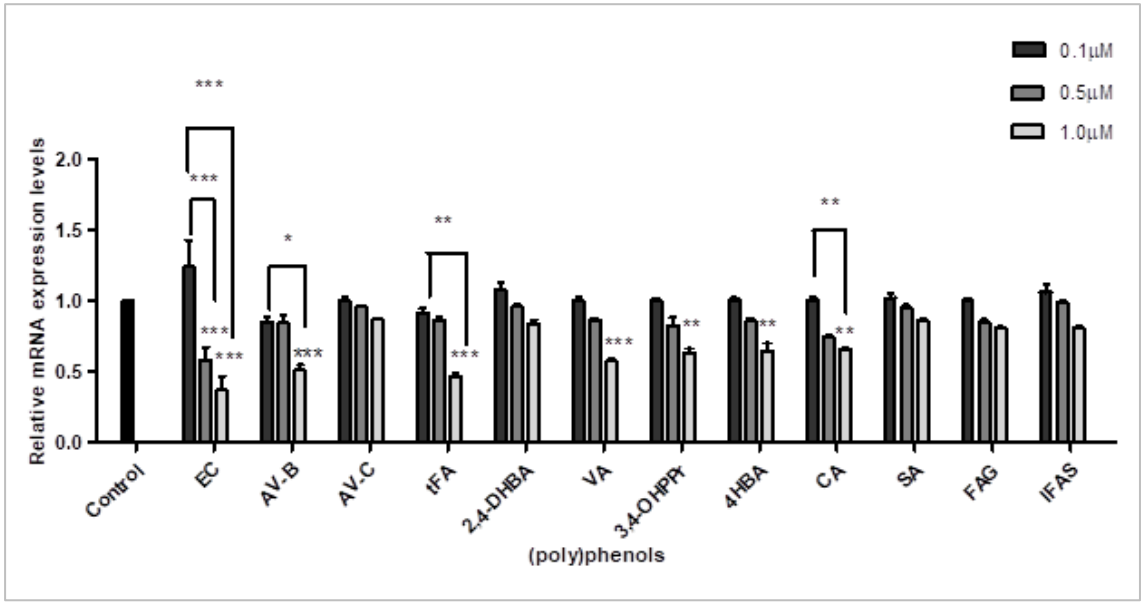


Figure 2.3 Effects of 24 h exposure to selected polyphenols (on levels of renin mRNA expression in Juxtaglomerular cells (AS4.1 cells)

Graph of effects of 24 h exposure to selected polyphenols (see Section 2.2.1 for full names) at concentrations of 0.1, 0.5 and 1.0 µM, on levels of renin mRNA expression in Juxtaglomerular cells, determined using real-time RT-PCR. Renin expression values were normalised to GAPDH values, expressed as fold change from control cells grown without polyphenols by $2^{-\Delta\Delta CT}$ method (relative value 1.0). Data presented as mean ± SEM of three independent experiments.
* - P ≤ 0.05: ** - P ≤ 0.01: *** - P ≤ 0.001.

mRNA expression in a concentration–dependent manner (Figure 2.3).

EC at 0.5 and 1 µM concentrations significantly ($P < 0.001$) decreased renin mRNA expression by 41.7 and 62.7 % respectively. Further inhibitory activity was observed for AV-B at 1 µM by 49 % ($P < 0.001$), while t-FA exerted a marked downregulation of renin mRNA expression by 53 % ($p < 0.001$) compared to the control value. In addition, there were significant effects on renin mRNA levels after exposure to 4HBA, CA and 3,4-OHPPr at 1.0 µM concentration by 35, 34.4, and 36.7 %, respectively ($p < 0.01$). After treatment with VA at 1.0 µM approximately 50-fold inhibition in renin mRNA expression compared to the control ($P < 0.001$), while, 2,4-DHBA, SA and AV-C at 1.0 µM induced non-significant decreases in expression of renin mRNA of about 15 % ($p = 0.527$, $p = 0.740$ and $p = 0.797$ respectively). Among the polyphenol compounds used, inhibition of renin mRNA expression following FAG and IFAS were lower than the others, statistics showed no significant difference compared to the control. No significant difference in gene expressions of renin mRNA was observed when the smallest concentration (0.1 µM) of any polyphenol was used.

2.3.4 Effects of Polyphenols on the Expression of CREB, ATF-1 and ERK1/2 Proteins

Levels of CREB, ATF-1, ERK1/2 protein expression were measured using western immunoblotting (as described in Section 2.2.8). AS4.1 cells were treated with selected compounds at 1.0 µm concentration for 1 hour. Calculated means of replicates normalised to total protein showed that relative levels of pCREB/total CREB protein were significantly reduced following all polyphenol treatments ($P \leq 0.001$) except 3,4-OHPPr (Figure 2.4). In addition, all polyphenol compounds tested induced a significant decrease in ATF-1 phosphorylation level when compared to total CREB (total CREB Mouse mAb also cross reacts with ATF-1) ($P < 0.001$) (Figure 2.5). With the exception of 2,4-DHBA, SA and AV-C, the polyphenols tested significantly ($P \leq 0.001$) affected the levels of ERK1/2 (Figure 2.6), with SA very close to a significant level ($P = 0.051$).

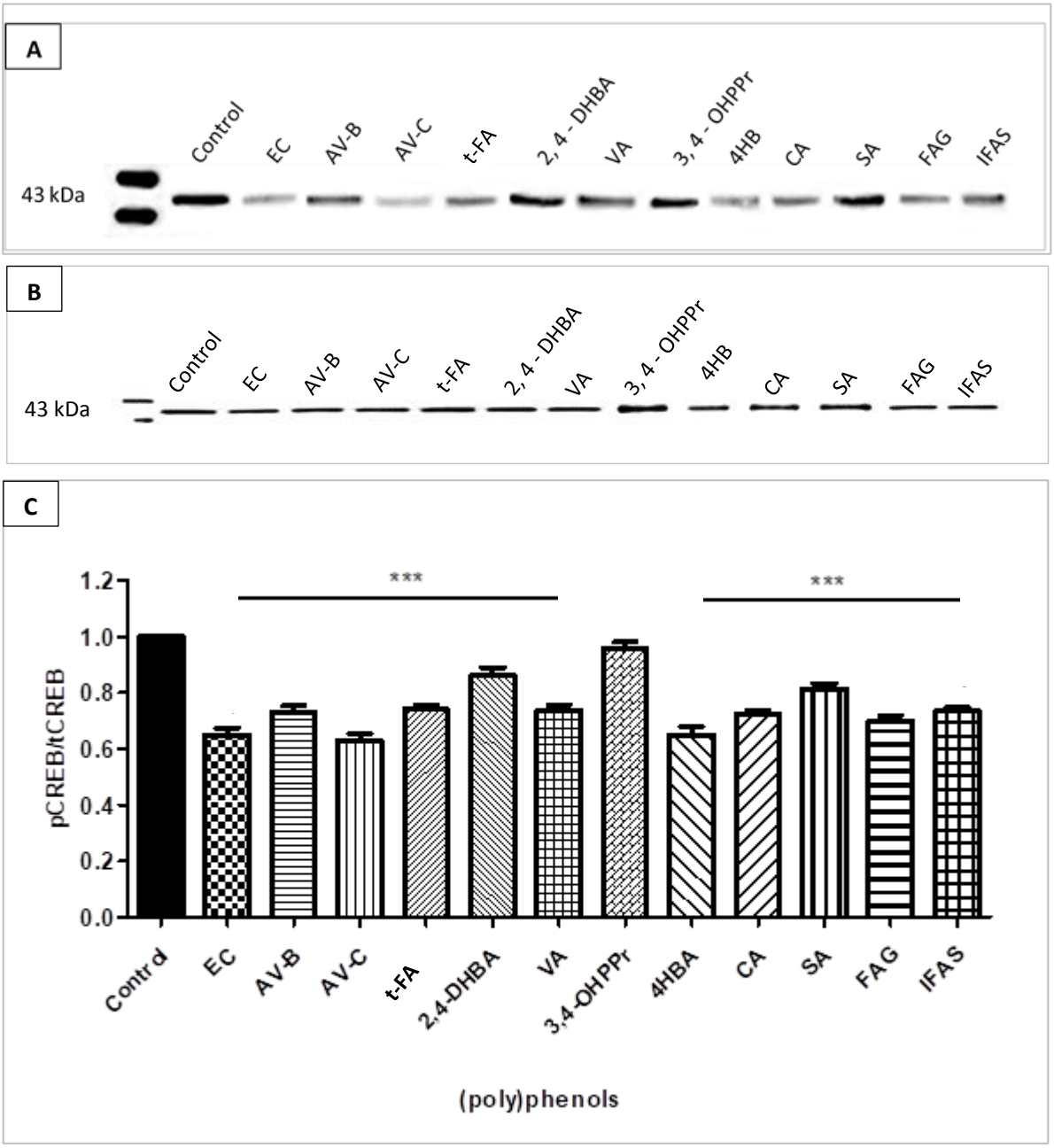


Figure 2.4 Effects of selected polyphenols (1 µM) on levels of CREB (Ser 133) in AS4.1

Cells were incubated with selected polyphenols (see Section 2.2.1 for full names) for an hour and determined by Western immunoblotting.

A. Phospho-CREB (ser133). **B.** Total CREB-1 protein. **C.** The bar diagram shows levels of Phospho-protein relative to total protein and expressed as a fold change from the control (relative value 1.0). Error bars are the SEM± of three independent experiments (n=3). * p-value ≤ 0.05 and ** p-value ≤ 0.01 and *** p-value ≤ 0.001.

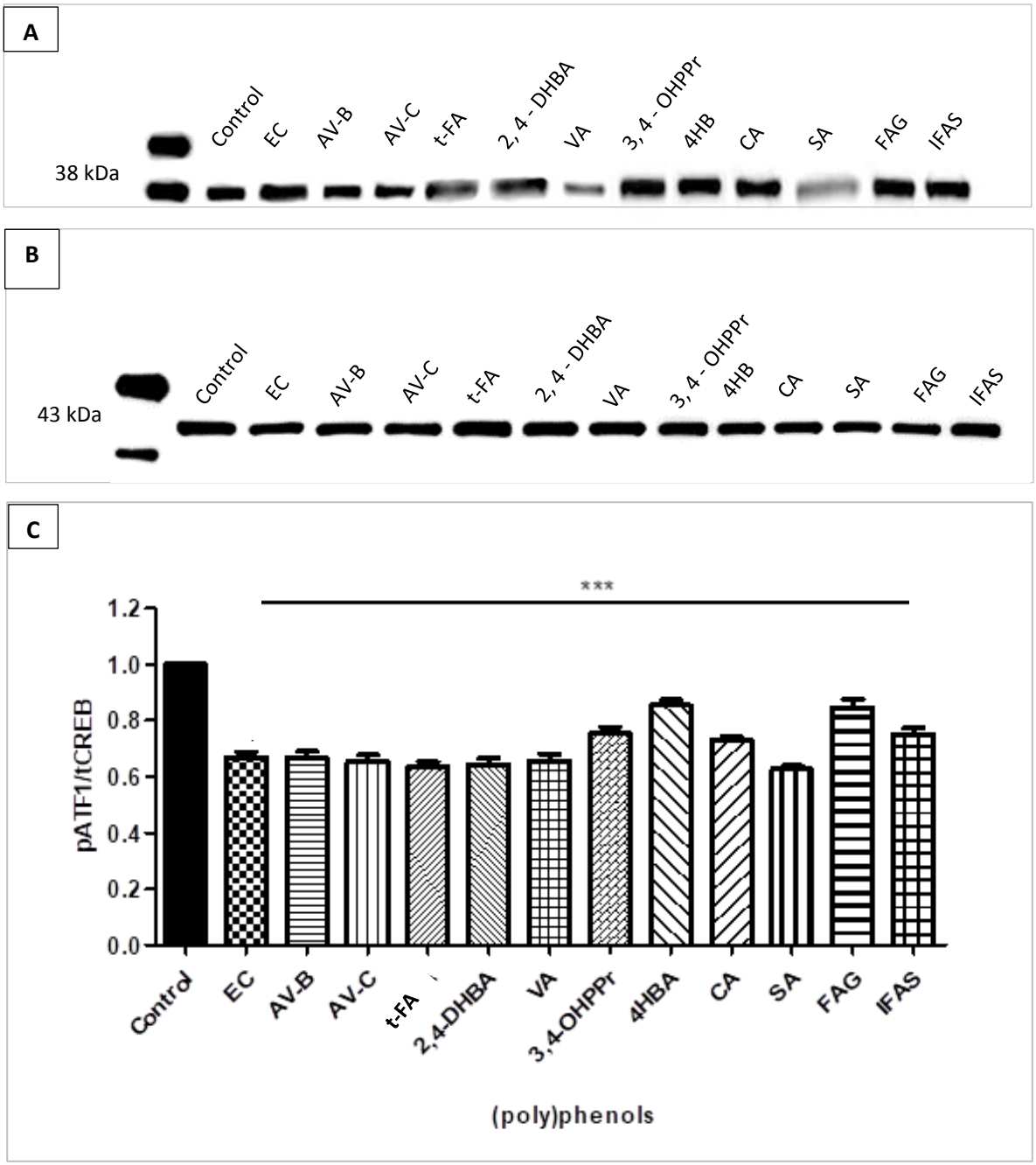


Figure 2.5 Effects of selected polyphenols (1 µM) on levels of ATF-1 (Ser 63) in AS4.1

Cells were incubated with selected polyphenols (see Section 2.2.1 for full names) for an hour and determined by Western immunoblotting.

A. Phospho-ATF-1(Ser63) protein. **B.** Total CREB-1 protein, used as control (total CREB Mouse mAb also cross reacts with ATF-1). **C.** The bar diagram shows levels of Phospho-protein relative to total protein and expressed as a fold change from the control (relative value 1.0). Error bars are the SEM± of

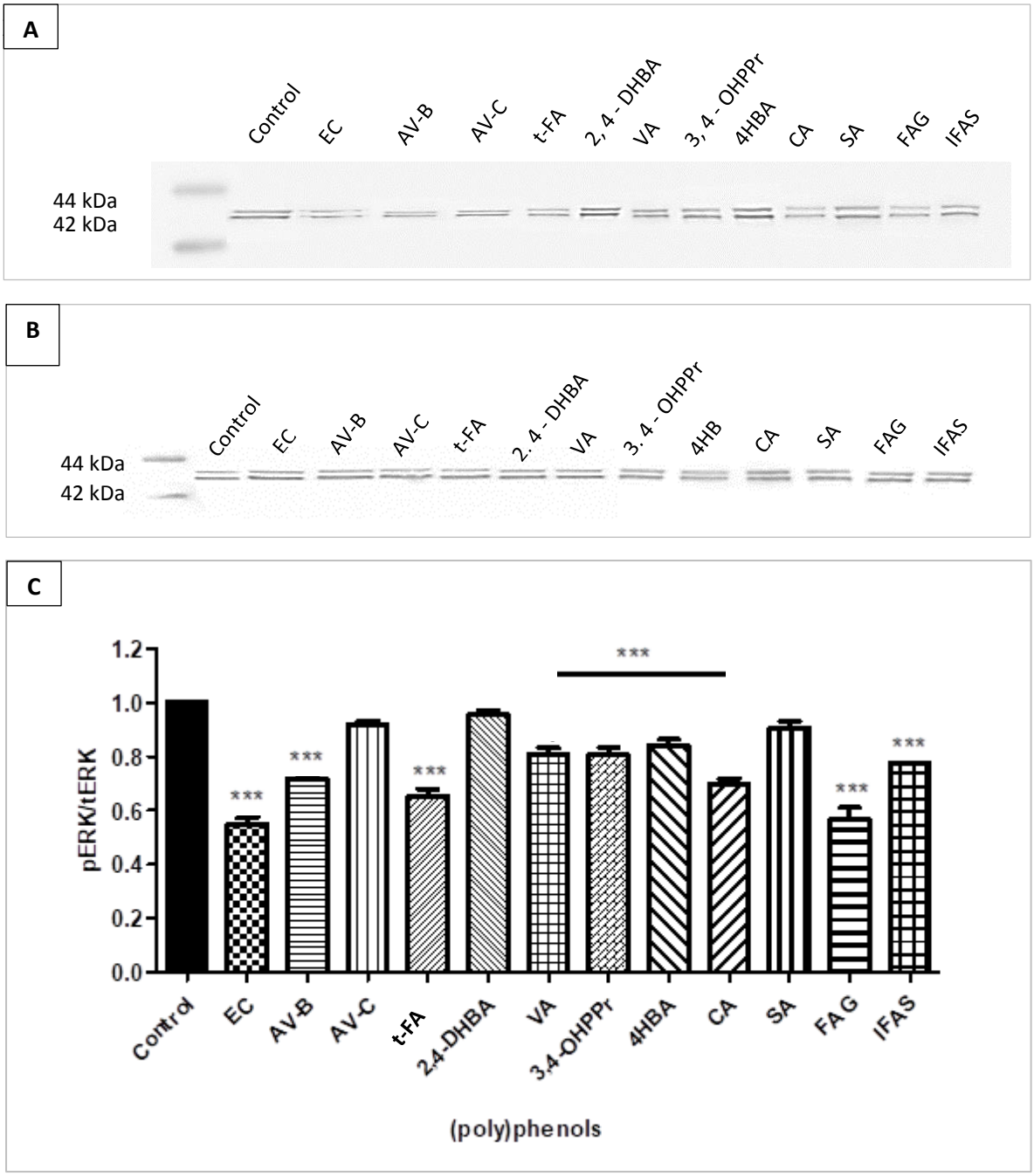


Figure 2.6 Effects of Selected Polyphenols (1 µM) on levels of ERK1/2 (Thr202/Tyr204) in AS4.1 Cells

Cells were incubated with selected polyphenols (see Section 2.2.1 for full names) for an hour and determined by Western immunoblotting.

A. Phospho-p44/42 MAPK ERK1/2 (Thr202/Tyr204). **B.** Total p44/42 MAP kinase (Erk1/Erk2). **C.** The bar diagram shows levels of Phospho-protein relative to total protein and expressed as a fold change from the control (relative value 1.0). Error bars are the SEM ± of three independent experiments (n=3). * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$.

2.3.5 SHED AND MEMBRANE-BOUND ACE

The measurements of shed ACE in HUVEC cells supernatant after 24 h exposure to different tested polyphenols at 1.0 µM concentration was not significantly changed when compared with the controls (cells without polyphenol treatment). Shed ACE was slightly increased following exposure to t-FA, although not significantly, whereas there was a decreased level of shed ACE following incubation with all other tested polyphenols (Figure 2.7). In line with this, ACE expression levels on the surface of cultured cells (membrane-bound ACE) were not affected by polyphenols when cells were incubated with the various polyphenols at 1 µM (Figure 2.8). Overall, tested polyphenols induced a weaker impact on ACE levels in membrane or in cytoplasm of endothelial cell in comparison to the control (calculated as percentage of non-treated control cells).

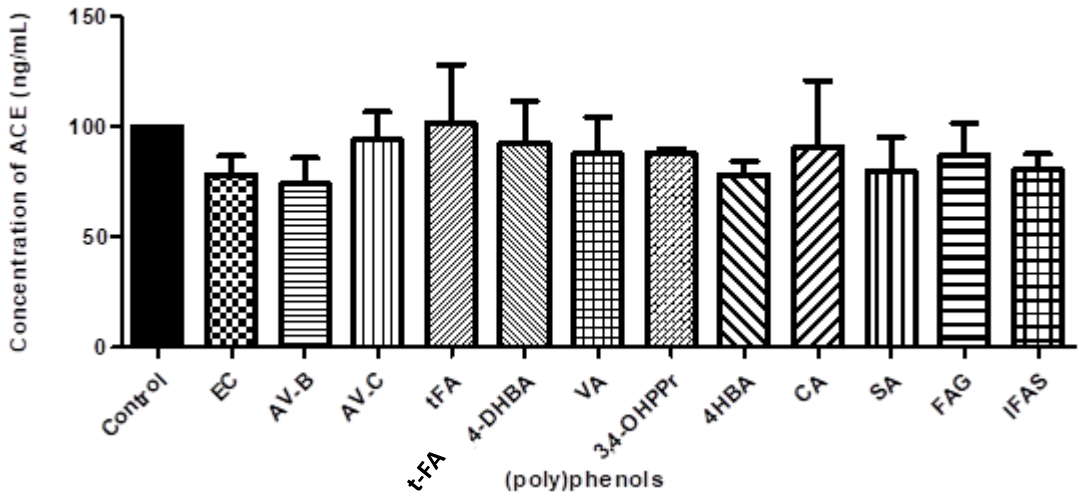


Figure 2.7 Detection of shed ACE in supernatant of HUVEC cells 24 h after treatment with selected polyphenols at 1.0 µM

Graph shows selected polyphenol (see Section 2.2.1 for full names) at a concentration of 1.0 µM in triplicate calculated as percentage difference from the non-treated control (100 %). Data presented as mean ± SEM.

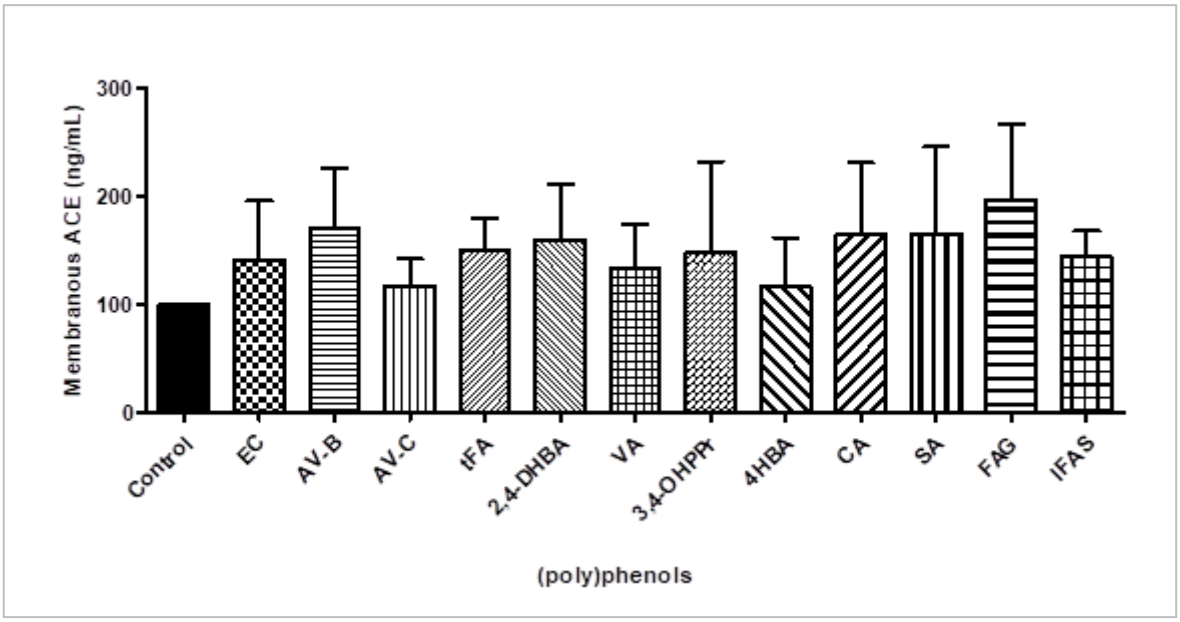


Figure 2.8 Effect of selected polyphenols on membrane bound ACE expression on the surface of HUVEC cells at 1.0 µM.

Graph shows surface labelling of ACE measured 24 h after treatment with selected polyphenols (see Section 2.2.1 for full names) at 1.0 µM, in triplicate, calculated as percentage difference from the non-treated control (100 %). Data presented as mean ± SEM.

2.3.6 ACE ACTIVITY INHIBITION

The ability of selected polyphenol compounds to inhibit ACE activity was also assessed. ACE activity was measured fluorometrically with Hip-His-Leu as substrate in homogenates of HUVEC cells. All tested polyphenols at 1.0 µM incubated for 24 h induced some inhibition of ACE activities ranging from 17.2 to 48.8 % compared to 100 % non-inhibited control. 2,4-DHBA, EC and SA inhibited ACE by 44.7 41.4 and 41.3 %, respectively. However, greatest inhibition was achieved by t-FA at 48.8 % ($P = 0.067$). ACE inhibition was not statistically significant for any of the tested polyphenols (Figure 2.9).

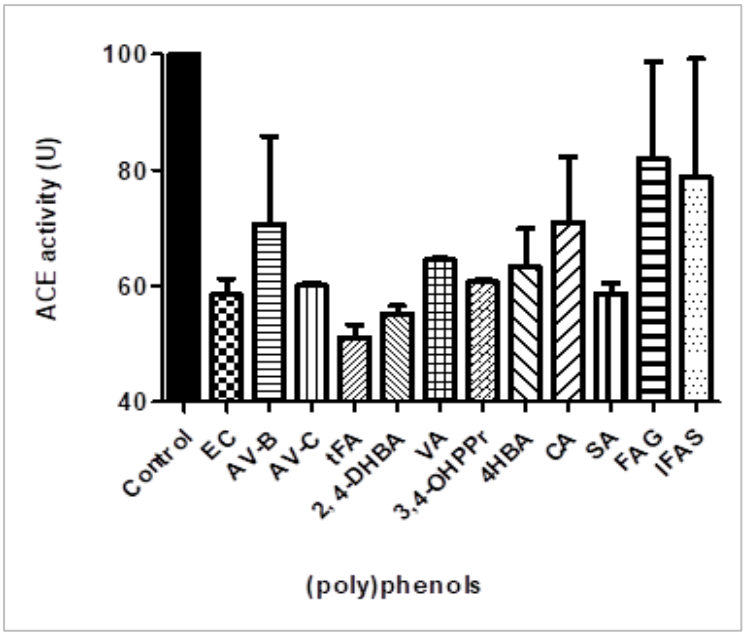


Figure 2.9 ACE activity in HUVEC cells 24 h after treatment with selected polyphenols at 1.0 µM.
Graph shows selected Polyphenols (see Section 2.2.1 for full names) applied at 1.0 µM, in triplicate, and calculated as percentage difference from the non-treated control (100 %). Data presented as mean ±SEM.

2.3.7 NO, Nitrite and Nitrate Analysis

Total nitrate and nitrite concentrations were analysed with a commercially available nitrite/nitrate assay. Following cell exposure to different polyphenols for 24 h, results indicated that NO was not significantly increased. There was even a slight decrease in total NO production with AV-B (Figure 2.10 (A)). The same was observed for nitrate, with no increase in nitrate levels and with a slight decrease with AV-B (Figure 2.10 (B)). An increase in nitrite production was seen with most polyphenols, except 2,4-DHBA, VA and FA, where a reduction was observed (Figure 2.10 (C)). None of the measured effects of polyphenols significantly ($P \leq 0.05$) affected total NO, nitrite or nitrate (Figure 2.10 (A-C)).

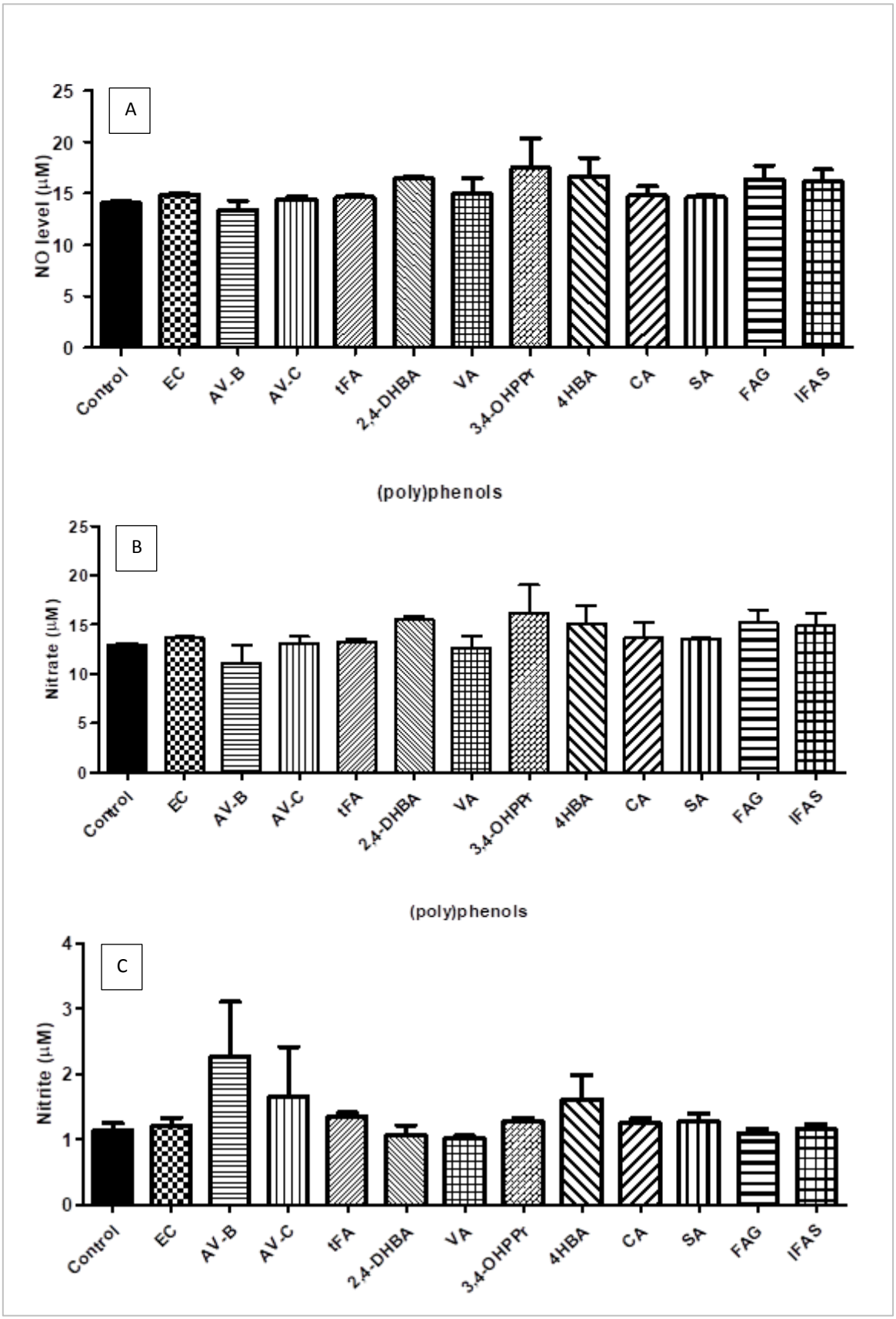


Figure 2.10 Total NO (A), nitrate (B) and nitrite (C) production in HUVEC cells after 24 h incubation with selected polyphenols at 1 µM

Selected (see Section 2.2.1 for full names) polyphenols at a concentration of 1 µM in triplicate, compared to control cells grown without polyphenols. Data presented as means ± SEM.

2.4 DISCUSSION

Several studies have shown that polyphenols improve vascular function and have potential cardiovascular health benefits [48, 49, 547, 548]. Polyphenols have been reported as a promising substitute for oral drugs that exhibit antihypertensive action [252, 499, 506, 549-551]. Modulation of the RAAS, a critical regulator of BP and vascular function, is one of several proposed mechanisms for their action, which may be affected by either inhibited renin expression or ACE activity or both [254, 297, 358, 362, 366, 369]. The aim of this study was to better comprehend the mechanism of action through which polyphenols achieve such physiological improvements, by investigating if they are able to influence the RAAS. The RAAS plays a crucial role in controlling BP via production of a vasoconstrictor, Ang II, which has a role in regulating blood volume and vasodilation independently of NO [501, 502]. Renin is the rate limiting step in the RAAS, hence it is believed to be a good target for treatment of hypertension and cardiovascular disease [552]. Likewise, suppression of renin and ACE are two of the strategies used for lowering BP, maintaining electrolyte homeostasis, and for haemodynamics and blood volume status regulation [253, 504].

We examined the *in vitro* effects of a range of common phenolic compounds on the RAAS at concentrations of 0.1, 0.5 and 1.0 μM. Trans-ferulic acid (t-FA) possessed the strongest renin inhibitory activity, with a mean IC_{50} of 0.51 μM, the proportion of inhibition varied from 6.43 - 79.81 %. Other polyphenols from different food sources have previously been shown to modulate the RAAS system, by inhibiting renin *in vitro*, including (-)-epicatechin gallate (IC_{50} = 619.4 μM), (-)-epigallocatechin gallate (IC_{50} = 44.5 μM) and (-)-epigallocatechin (IC_{50} = 2175.3 μM) [499]. Studies using tea polyphenols including theasinensin B (IC_{50} = 19.33 μM), theasinensin C (IC_{50} = 40.21 μM), strictinin (IC_{50} = 311.09 μM), and a hexose sulphate (IC_{50}= 50.16 μM) have also demonstrated the suppression of renin activity [553]. Renin suppression may have been due to the presence of polyphenol derived special functional structures such as analogues of galloyl moiety without a catechin skeleton and ortho-tri-hydroxy phenyl. Polyphenols may conform the renin active site, reducing its activity, by forming an enzyme-substrate-inhibitor complex. However, it is also possible that the complex biochemical transformations that the numerous polyphenols in plants, for example those in tea, undergo during the fermentation process which is typically part of its preparation for consumption, form a variety of bioactive substances, including *inter alia* theasinensin B, theasinensin C and a hexose conjugate. Previous studies have reported that polyphenols especially gallated flavonoids are able to inhibit renin activity. However, there is no evidence that any phenolics inhibit renin activity.

In order to better understand how polyphenols influence renin expression, we also explored their effects on renin gene expression in juxtaglomerular cells (AS4.1). Renin gene expression was significantly decreased following exposure to EC, AV-B, t-FA, 4HBA, CA, 3,4-OHPPr and VA at higher concentrations relative to the control ($P < 0.01$ to $P < 0.001$, in all cases). In mouse renin-expressing clonal cell line derived from the kidney cells (As4.1), which have many features characteristic of the human kidney renin-expressing juxtaglomerular cells, it has been shown that site D contains a CRE, which binds several transcription factors including cAMP-response element-binding protein (CREB) and ATF1. A pathway has been identified which regulates the expression of the renin gene, mediated by both cAMP-responsive element binding protein-1 CREB-dependent and CREB-independent mechanisms, in Calu-6 cells [509, 512]. Further, in renin expressing cells, the binding of five specific DNA-protein complexes, consisting of the ATF-1 and CREB-1 transcription factors, one of which was an ATF-1zCREB-1 heterodimer, suggests the potential for regulation of CREB-1 activity by ATF-1. Therefore the cAMP-PKA signalling pathway in renin expressing cells transcriptionally activates human renin promoter [512]. Therefore, our hypothesis was that polyphenols might be capable of modulating the synthesis of renin, by interacting with the activation of CREB-1 and ATF-1 proteins, and indeed ERK1/2, which are upstream activators of CREB and ATF-1. The phosphorylation and activation of all proteins were significantly reduced following polyphenol treatment ($P \leq 0.001$, in all cases) in comparison to total protein in control cells. CREB/ATFs are known to mediate stimulatory effects on renin synthesis in juxtaglomerular cells [508, 510, 511]. Since CREB expression was observed to be inhibited by several polyphenols in oats, it is likely that this may be one mechanism by which renin synthesis is decreased post incubation with polyphenols. Previous data supports nutrient and non-nutrient inhibition of renin expression. One study showed that vitamin d3 (calcitrol) suppresses the transcription of the renin gene through blocking the formation of the CRE-CREB-CBP complex in the renin gene promoter [554]. In another study involving As4.1 cells, it was shown that IL-1β downregulates renin gene expression via a mechanism involving the Erk-STAT3 pathway. IL-1β-plays a role in phosphorylation of ERK1/2, which in turn suppresses renin by upregulation of STAT-3 [555]. Therefore, reduction in renin gene transcription in juxtaglomerular cells post incubation with some polyphenols might be a result of inhibitory interactions with CREB, ATF-1 and ERK 1, 2.

Several studies have shown that polyphenols influence ERK and Protein Kinase B (Akt) signalling pathways [556, 557]. For example, flavonols and anthocyanins in blueberry modulate the ERK-CREB-BDNF pathway in rodents [558]. In another study, quercetin and its O-methylated metabolites have been shown to induce neuronal death by inhibition of neuronal survival

signalling through inhibition of ERK instead of activating the c-Jun N-terminal kinase-mediated death pathway [556]. There is also substantial evidence that the phosphorylation of CREB, and its subsequent interference with the CRE, activates renin gene transcription in juxtaglomerular cells, include AS4.1 and Calu-6 cells [509, 512, 515, 559-561]. In conclusion, it is widely reported that polyphenols interact with the ERK-ATF-1 pathway and P38/ CREB, to reduce expression of the renin gene, and that this leads to improved cardiovascular health [558, 562, 563].

We also explored the effect of selected polyphenols on ACE and NO using HUVEC cells and found most inhibited ACE, but to different degrees and none was significant; t-FA showed the strongest inhibitory effect. ACE inhibition by polyphenols has been demonstrated in similar cell strains by tea polyphenols. ACE activity, and substrate-dependent reaction kinetics have revealed enzyme velocity curves that matched allosteric and non-Michaelis-Menten relationships, with a mixed mode of *in vitro* inhibition of ACE, mostly of a kinetically uncompetitive type [362], by green and black tea polyphenols. Further, studies on cultured endothelial cells from human umbilical veins incubated with a bilberry polyphenol extract exhibited significant, also inhibited ACE activity after 10 minutes, dose dependently [369]. These findings were not supported in a study on rats [564] which found that ferulic acid lowered ACE activity by the greatest amount (18 %). The significance of this result may be due to the use of a higher dose, 9.5 mg/kg *in vivo* compared to our 1.0 µM *in vitro*, and to the use of an animal model which is more similar to human essential hypertension than our *in vitro* model [564]. Other phenolic compounds have been identified as ACE inhibitors *in vitro* including vanillic acid, ferulic acid, p-coumaric acid, caffeic acid, p-hydroxybenzoic acid and benzoic acid (IC_{50} = 8, 4.40, 2.80, 2.10, 5.95 and 6.20 mM respectively), which are also present in oats [565]. The mechanism of ACE activity inhibition is at least in part through interactions with amino acids at the active site, which stabilise phenolic compound interactions with zinc ions [566]. Previously reported studies have revealed that caffeic acid at a dose of 10 to 1000 µM helps in multi-target modulation of RAAS by inhibiting ACE activity (IC_{50} = 430 µM), caffeic acid also reduced aldosterone production [361, 566]. Six flavonoids, apigenin, luteolin, kaempferol-3-O-α-arabinopyranoside, kaempferol-3-O-β-galactopyranoside, quercetin-3-O-α-arabinopyranoside and luteolin-7-O-β-glucopyranoside have also been shown to inhibit ACE, with kaempferol-3-O-β-galactopyranoside the most active (IC_{50} = 260 µM) [567]. A 3% blueberry diet for 2 weeks, given to stroke-prone spontaneously hypertensive rats, lowered ACE activity but had no effect on normotensive rats, suggesting that managing the early stages of hypertension with dietary flavonoid-rich blueberries may be effective, partially through, they state, soluble ACE activity inhibition [568]. In another study, examining the mechanisms of the inhibitory activities of flavonoids and metabolites in flavonoid-rich apple peel extract, it was found that inhibition was

due to the presence of hydroxyl groups and the B ring, as quercetin-3-O-glucoside has significant ACE inhibitory action compared to that of quercetin [569]. In conclusion, there is, therefore, substantial evidence to show that polyphenolic compounds inhibit ACE activity. In our results, however, we measured smaller effects than many previous studies, this may be due to the differences in doses and polyphenol types, structures or concentrations, the different experimental models used, For example some previous published significant result from animal studies due to the duration of the study chronic study than single oral administration. However, our in vitro experimental conditions include concentrations and application for 24 h may have been influential in the weakness of the ACE activity responses and changes to NO levels. It is also possible that pure phenolic compounds could not fit the active site of ACE, due to their too large polymer sizes. However, it should be noted that the unaltered polyphenols hexameric phenol and dieckol, have been shown to inhibit ACE by binding on the outside of the ACE molecule [570]. Furthermore, phenolic acids and flavonoids, such as quercetin, are reported to inhibit ACE activity via interaction with the zinc ion in the active site. Metalloenzymes, including zinc metallopeptidases, are known to be inhibited by suitably hydroxylated flavonoids and other polyphenols [571, 572]. It was also concluded that the inhibitory capabilities of phenolic compounds increased as their number of hydroxyl and acrylic acid groups increased, hence salidroside, with only one hydroxyl and no propenoic acid, compared to the ortho-dihydroxyl and propenoic acid properties of caffeic acid, showed much lower ACE inhibiting activity. Another substructure of phenolic acids that seems to influence ACE inhibitory activity, albeit negatively, is the methoxy functional group. This may be due to their steric hindrance to binding to the ACE active site [566]. It has become apparent that the presence of certain functional groups, such as hydroxyl, carboxyl, and acrylic acid, which can act as hydrogen bond acceptors or donors, seems to increase the potency of polyphenols to inhibit ACE, which suggests that it is not simply the presence or absence of such groups that influences the potential of phenolic acids to inhibit ACE activity. Studies have revealed, further to this general situation, that most phenolic acids form charge−charge interactions with the zinc ion at the active site of ACE, via the oxygen atom in the carboxylate moiety. The exact mechanism by which t-FA lowers ACE activity remains, however, unknown.

Cell exposure to 1 μM of various polyphenols for 24 hours led to non-significantly increased total NO, nitrite and nitrate in every case, except a slight decrease in total NO production was seen for AV-B and a reduction in nitrite following 2,4-DHBA, VA and FAG exposure. These results were not fully reflected in a study which found that Avenanthramide-2c, a polyphenol, found exclusively in oats, when applied at a concentration of 120 μM increased NO production three-fold in smooth

muscle cells and nine-fold in human aortic endothelial cells [406]. This may be due to the higher concentrations of Avenanthramide-2c used in comparison to ours. Other studies have revealed the highest release of NO, at Δ[NO] > 8.5 nM, is induced by quercetin, myricetin, leucocianidol and oligomeric proanthocyanidins while moderate release, Δ[NO] > 5 nM < 8.5 nM, was shown after caffeic acid, fisetin, hyperoside and isoquercitrin and only marginal release after other phenolic compounds, Δ[NO] < 5 nM [573]. In this study a flavan moiety with free hydroxyl-residues at C_3, $C_{3'}$, $C_{4'}$, C_5, and C_7 and a hydroxyl-, oxo-, or phenolic substituent at C_4 was associated with high NO activity. The NO–stimulating and vaso-relaxing activities of the polyphenols were also positively correlated, hence the increase in NO due to polyphenols may promote cardiovascular health, but the concentration required for this effect is very high.

The reduced expression of ACE in pulmonary microvascular endothelial cells is caused by two processes: initial increased shed of ACE followed by a compensatory downregulation of ACE-mRNA and membrane-bound protein expression [574]. Hence, we performed similar trials with polyphenols, to determine if they also contribute to ACE expression inhibition by increasing either shed or membrane-bound ACE. The measured levels of shed ACE in supernatant from, nor membrane-bound on, HUVEC cells after 24-hour incubation with polyphenols (1.0 μM), were not, however, significantly changed. Therefore, we conclude that no substantive effects of polyphenols on ACE activity, whether membrane-bound or shed, were detected by our experiments.

We did find, however, strong evidence from our experiments to conclude that polyphenols do modulate renin gene expression through their effects on the CREB, ERK and ATF transcription factors. However, unlike some published studies, we failed show that polyphenols inhibit ACE activity significantly via this mechanism. We also found no significant increase in total NO, nitrite and nitrate post incubation with polyphenols at 1 μM, however, we measured inhibitions smaller than those reported in many previous studies. We, therefore, conclude that polyphenols may reduce BP via the RAAS, but need higher doses for ACE enzyme inhibition. Polyphenols at physiological concentrations could decrease BP, although we cannot explain the exact mechanism(s) of lowered renin expression or ACE inhibition and as very few studies have been published in this area, this remains an important gap in our knowledge. Further studies are needed to increase our understanding of the biochemical complexities of the relationship between polyphenols and inhibition of RAAS and decrease of BP.

Since these results were promising by clearly demonstrating that small phenolic compounds in physiological concentrations might affect the RAAS albeit minimally, they may explain why people

who consume foods rich in such polyphenols might experience a reduction in BP. Therefore, the next two chapters will seek to expand on these findings and provide a better understanding of the physiological effects of specific polyphenols through both acute and chronic clinical trials.

Chapter: 3 Investigation of the Vascular Effects of Acute Oat Intake on Males with Pre- and Stage 1 Hypertension

3.1 INTRODUCTION

The purpose of this investigation, of an acute oat intervention on the cardiovascular health of males with pre- and stage 1 hypertension, is to build on the large body of existing knowledge in this field, which has amassed from studies of polyphenols originating from other plant sources on individuals with the same or similar physiological conditions. This group of people is recognised to be particularly responsive to interventions which affect blood pressure (BP) and endothelial function. We anticipate that, as in comparable studies, physiological changes in men in this trail will occur following an acute polyphenol intervention, from oats in our case, with measurable effects on the RAAS, specifically in observable inhibition of ACE and renin. Our trials include several metrics commonly tested to represent improvements in BP and vascular function, notably Flow-Mediated Dilatation (FMD) and endothelium dependent and independent microvascular responses. ACE activity and ACE and renin concentrations were assayed using widely deployed Enzyme-Linked Immunoassays (ELISA). There are no comparable clinical trials which test the effects of oat specific polyphenols on BP and vascular function. The clinical vascular data which does exist applies instead either to wholegrain oats without, differentiating the impact of polyphenols from fibre, or else where polyphenols are specifically targeted, to a number of other phenolic-containing foods and beverages, some of which have been investigated repeatedly [335, 575-579].

Recent studies suggest that diets rich in polyphenols have a range of beneficial effects on cardiovascular health [47, 181, 241-244]. Polyphenol rich foods which have been studied include fresh fruits (particularly berries) [49, 335, 580], vegetables (red onions), whole grains (oat, wheat, rye), tea, coffee, cocoa [498, 581], nuts (chestnut, pecans, hazelnuts), red wine, seeds (flaxseed) , olive and spices (cinnamon, cloves), all of which can be included with relative ease into daily diets [247, 498, 582-588]. The ability of polyphenol-enriched foods to attenuate the incidence of CVD, has been attributed to their anti-thrombotic, anti-atherogenic and anti-inflammatory properties, as well as their potential to induce vasodilatation and to reduce blood pressure in hypertensive individuals [296, 403, 589, 590]. With respect to the two latter outcomes, their potential to influence two key pathways of blood pressure (BP) homeostasis, endothelial nitric oxide synthase (eNOS) and/or the Renin-Angiotensin Aldosterone System (RAAS), are likely to underpin their

actions at the physiological level. Nitric oxide is the most potent vasodilator in the body and any defect in its synthesis or activity may lead to endothelial dysfunction, signalled by impaired endothelium-dependent vasodilation. Impaired endothelium-dependent vasodilation is the common cause of vascular dysfunction, one of the most important, early markers of atherosclerosis [86]. The RAAS plays a crucial role in controlling blood pressure via production of a vasoconstrictor, angiotensin II, which has a role in regulating blood volume and vasodilation independently of nitric oxide (NO) [501, 502]. Studies have shown that acute consumption of flavanol-rich dark chocolate improved endothelium-dependent vasodilation, flow-mediated dilatation (FMD) and protected from vascular impairment induced by a glucose load [581, 591]. Similarly, acute interventions with cranberry polyphenol metabolites and flavonoid-rich acai meals are both associated with increased FMD and vascular function [335, 592]. Flavonol-rich grape juice containing cinnamic acid, decreases platelet aggregation, that cranberries rich in anthocyanin reduce BP and heart rate, and that pomegranate juice rich in tannins and anthocyanin induce vasodilation [186]. Small phenolic compounds such as hippuric, vanillic, ferulic and homovanillic acids have been recognised as structurally homologous to NAPDH oxidase inhibitors present in endothelial cells [593]. Likewise, betulinic acid reduces nitric oxide activity via upregulation of endothelial NO synthase (eNOS) and down-regulation of NADPH oxidase [594].

Whole grain cereals, while well known for being high in fibre, are also a rich source of other macro- and micro-nutrients and non-nutrients, including phenolic acids. Oat (*Avena sativa* L.) grains are rich in unsaturated fatty acids and soluble fibres (β-glucan), as well as phenolic acids such as ferulic acid and phenolic amides called avenanthramides [565, 595]. It has been postulated that these small phenolics may contribute to reductions in BP and blood cholesterol, and increases in nitric oxide thus contributing to a reduction in CVD risk [40, 406]. Avenanthramides, exclusively present in oats, have been reported to be bioavailable, and shown previously to inhibit the development of vascular smooth cells, in *in vitro* studies, and presented potential health benefits by acting against the adverse effects of atherosclerosis [405, 406, 596].

Studies have shown that oats could reduce systolic blood pressure (SBP), pulse pressure and improved vasodilation [53, 597]. The addition of whole oats to daily diets of hypertensive individuals led to reductions in total cholesterol and low-density lipoprotein cholesterol by at least 10 % within 6 weeks and a reduction in systolic and diastolic BP [598]. It has been speculated that the inclusion of whole oats in the diets of middle-aged men and women may to lower BP and lipids and so contribute to reducing the risk of CVD by circa 30 % [599, 600]. The cardiovascular benefits of whole grain oats have been quite widely investigated and found to mediate vasodilation [487, 601]. Oat bread was shown to induce a significant increase in baseline brachial

artery diameters and post-ischemia diameters [602]. Oat consumption may attenuate declines in brachial artery flow responses induced by high fat intake [603]. Green oat extracts, derived from blending whole, young oat plants, were reported to improved vasodilator function of systemic and cerebral arteries [53]. The American Heart Association and other international health associations now recommended dietary fibres, and whole grain cereals such as oats, to help achieve overall goals of implementing a heart-healthy diet [604, 605]. Appropriate studies, therefore, to investigate and validate the association of whole grain oats and its constituents with cardiovascular benefits may further help to present oats as a valuable and sustainable dietary option, especially for adults in prehypertension.

This study tests the hypothesis that a single intake of oats per day can result in positive CVD benefits for healthy men with prehypertension or stage 1 hypertension, as demonstrated by improvements in CVD markers, including vascular functions, BP and RAAS enzymes inhibition, and that such changes may be, in part, regulated by concentration of avenanthramide and phenolic acids.

3.2 MATERIALS AND METHODS

3.2.1 SELECTION OF SUBJECTS

Potential volunteers were recruited using the Hugh Sinclair Unit of Human Nutrition database and local advertisements. Interested volunteers were given a participant information sheet (Appendix 1) detailing the background of the study and what was expected of them. Volunteers still interested in participation were asked to fill in a health and lifestyle questionnaire (Appendix 2) to verify that they met the broad inclusion/exclusion criteria, for example age, dietary allergies and medication intake, as outlined below.

Volunteers eligible for inclusion, measured as described below, comprised: males, 25-65 years old, pre- or stage 1 hypertension SBP 120-159 mmHg and DBP 75-99 mm Hg), non-smoking, not taking any long-term medication on the exclusion list below, free of disease and not undertaking vigorous exercise daily. The exclusion criteria included: individuals who were hypertensive (SBP/DBP/≥ 160/100 mm Hg), BMI > 35, current smoker or ex-smoker, allergic to whole grains and taking any BP, anti-inflammatory, antidepressant, antibiotic or blood fat medication.

Volunteers meeting the broad criteria attended a short screening visit, after having fasted overnight (i.e. no intake of food or drink for 10 h before the visit, except water), where the study was explained in detail and they were encouraged to ask any remaining questions. Anthropometric measures, blood pressure measurements and 20 ml of blood were collected.

Blood samples were analysed for biochemical and haematological markers partly on site at the University of Reading and partly by the pathology department of the Royal Berkshire Hospital (Reading, UK). Based on screening results, as detailed below, 16 suitable individuals were invited to participate in the study. All volunteers provided written, informed consent before commencing the study (Appendix 3).

3.2.2 STUDY DESIGN

The study was approved by the University of Reading Research Ethics Committee and registered at www.clinicaltrials.gov as NCT02731755.

The proposed study was a single-centre, two-arm, single-blinded, placebo-controlled randomised crossover trial. Volunteers were required to attend the Hugh Sinclair Unit of Human Nutrition on two occasions during which they either consumed one portion of avenanthramide and phenolic acids-rich oats or an energy matched control intervention, in random order. The primary expected outcome was flow-mediated dilatation of the brachial artery (FMD). Secondary outcome measures were microvascular endothelial function (measured by Laser Doppler Iontophoresis), BP changes, level and activity of the renin angiotensin system.

3.2.3 STUDY INTERVENTION MATERIALS

The study intervention materials comprised porridge containing 90.2 g oats (67.7 g CDC dancer : oat flakes and 22.5 g oat bran concentrate) (Pepsico, Barrington, USA) and 420 ml water. This provided 45.0 mg of phenolic acids and 5.0 mg avenanthramides (total 50.0 mg). The control intervention consisted of cream of rice (B&G Foods, Inc.), dry skimmed milk, sunflower oil, cellulose powder and pectin powder containing 4.2 mg phenolic acids and was closely matched to the porridge for macro- and micro-nutrient content (Table 3.1). β- glucan, betaine, choline and trigonelline were present at levels too low to induce any observable vascular effects [602, 606].

Oat interventions were measured by HPLC for phenolic acid content and analysed for macro and micronutrients by Campden BRI (Gloucestershire, UK). All dry materials were frozen until needed to prevent degradation and prepared as detailed in Appendix 4. Both foods were well tolerated by all subjects, and no adverse events were reported.

	Oat intervention [1]	Control intervention [2]
Phenolic acids (mg)	45.0	4.2
Avenanthramides (mg)	5.0	0.0
Energy (kcal)	322	322
Fat (g)	6.3	6.3
Saturated fatty acids	1.2	0.9
Monounsaturated fatty acids	2.2	1.3
Polyunsaturated fatty acids	2.6	3.9
Carbohydrate (g)	47.1	47.1
Fibre (g)	12.1	12.1
Beta-glucan	5.6	0
Pectin	0	5.6
Protein (g)	13.3	13.3
Betaine (mg)	25.2	1.5
Choline (mg)	12.0	2.6
Trigonelline (mg)	34.0	2.0

Table 3.1 Study intervention materials

Polyphenols are highlighted in blue.

[1] 67.7g CDC dancer oat flakes, 22.5g oat bran concentrate and 420ml water.

[2] 39.4g cream of rice, 6.1g sunflower oil, 29.5g skimmed milk, 5.6g pectin powder, 6.5g cellulose and 420ml water.

3.2.4 RANDOMISATION AND MASKING

To minimise bias researchers was 'blinded' to the food (intervention) product. Scientists not involved in the study generated random number sequences, and managed volunteer allocation and intervention meal administration. Volunteers were randomly allocated to the intervention sequence (i.e. experimental intervention followed by control intervention or vice versa).

3.2.5 STUDY VISITS

Each eligible volunteer was asked to attend 2 identical assessment visits, separated by at least 1 week, at the Hugh Sinclair Unit of Human Nutrition. At each visit, the acute effects of a single intake of phenolic-rich oat porridge or control intervention was assessed. Volunteers were asked, in preparation for the visit, to fast overnight (i.e. no intake of food or drink for 10 h before the visit, except water), maintain complete dietary record sheets and to restrict their diet as detailed in Appendix 5, which included refraining from consuming (poly) phenol-rich foods, moderating consumption to ≤ 21 units of alcohol/week and to refrain in exercise 48 h prior to the study visits.

On arrival for a study visit, volunteers completed a researcher-administered questionnaire to monitor any potential adverse events and changes in medication, and to collect a full dietary record from dietary record sheets and to verify adherence to the lifestyle restrictions. Volunteers

were then rested for 15min in a supine position in a temperature-controlled (22 ± 1 °C), dimmed room, after which a series of non-invasive clinical measurements were taken, and blood and urine samples collected and immediately processed, following standardised procedures.

After completion of baseline measurements, a researcher not directly involved in the study provided the intervention products, which the volunteers were asked to consume completely over a 15min period. Up to 24 h post-intervention, non-invasive clinical measurements and blood and urine sample collections, as detailed below, were continued at set times (i.e. baseline, 1, 2, 4, 6 and 24 h; Figure 3.1). Between the 6 h and 24 h assessments, volunteers were allowed to leave the facility. To ensure the volunteers' well-being, they were encouraged to keep well-hydrated during the study by drinking enough water, while otherwise consuming just the phenolic-free meals as provided. Phenolic-free meals comprised a cheese sandwich consumed 5 h after the study intervention, and a main meal of pasta bake and crème brûlée which volunteers took home and consumed 8 h after the study intervention. Water intake throughout the day was recorded. A further light meal was also provided at the end of the assessment visit.

3.2.6 BLOOD COLLECTION

Blood samples were collected into vacutainers containing lithium heparin serum isolation's anticoagulants, EDTA and no serum isolation anticoagulants (Greiner Bio-one, USA), using standard venepuncture procedures. Thereafter, the vacutainers were kept in ice for lithium heparin and EDTA or stored at room temperature for serum separator vacutainers and allowed to coagulate for 30-60 minutes before centrifugation for 15 min at 3000 g at 4 ˚C, in order to separate the plasma or serum. The serum and plasma were isolated and stored in screw cap microtubes at -80 ˚C.

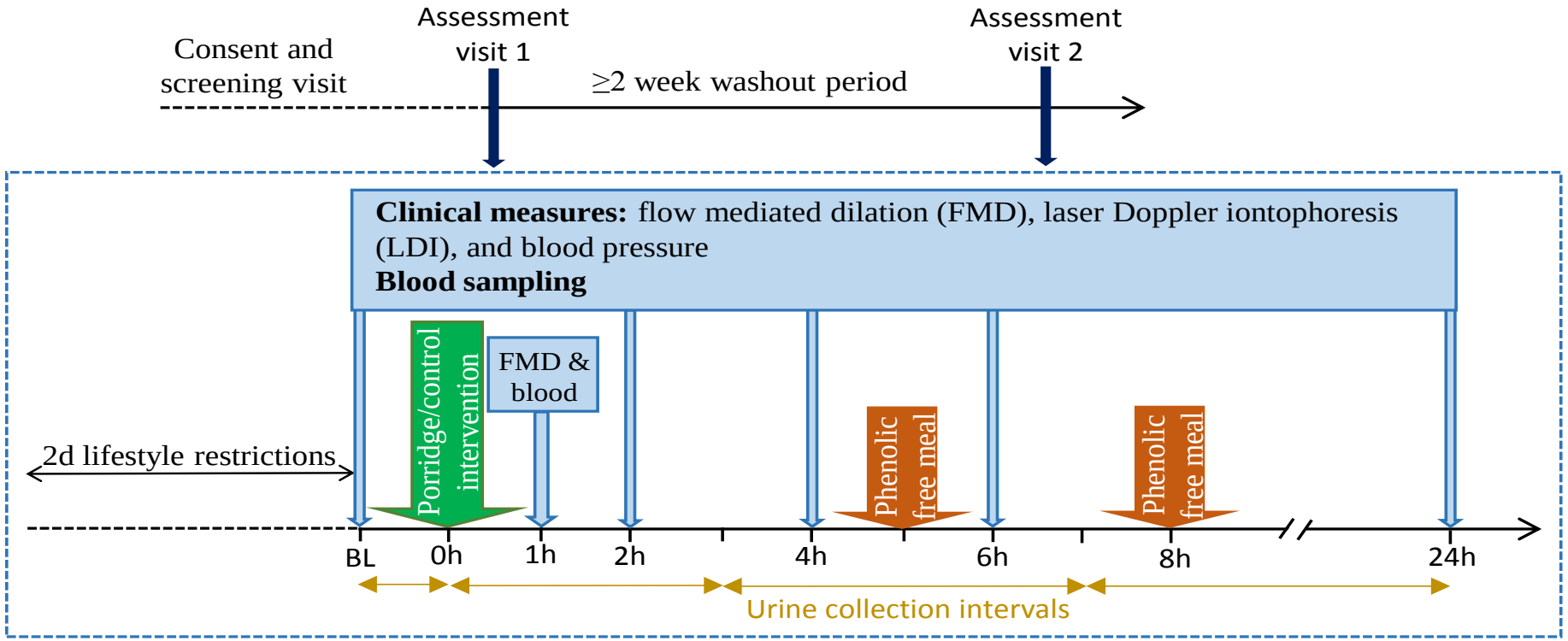


Figure 3.1 Outline study design

24 hour intervention periods and assessments at the Nutrition Unit. Interventions and assessment times are shown.
BL – Baseline.

3.2.7 OUTCOME ASSESSMENTS

3.2.7.1 FLOW-MEDIATED DILATATION

Endothelial-dependent vasodilation was measured by using an ATL Ultrasound HDI-15000 system (ATL Ultrasound) following standard guidelines [607]. Briefly, by using an electrocardiogram gated images (ECG-gated trigger) and image-grabbing software (Medical Imaging Applications-llc) to collect images. After a supine rest lasting 15 minutes in a quiet, temperature-controlled room, the brachial artery 2-5 cm above the antecubital fossa was imaged collected at 0.25 frames/s. Doppler-derived velocity of arterial blood flow was measured for 1 min prior to commencing each FMD measurement. Baseline images were taken for 1 min, after which the BP cuff inflates to 220 mm Hg to occlude blood flow. A sphygmomanometric cuff is placed just under the elbow of the non-cannulated arm and inflated using a pressure of 220 mm Hg for 5 minutes to induce a restriction of blood flow in the brachial artery. The pressure cuff is then released to induce reactive hyperaemia and hence vasodilatation of the brachial artery. After 5 min of occlusion, the pressure is rapidly released, allowing reactive hyperaemia to occur; measurement collection continued for 5 min post release. Analysis of the images were performed using wall-tracking software (MIA-llc). Image files were analysed by a single researcher who was blinded to the measurement details, and peak diameter will be defined as the largest diameter obtained after the occlusion is released. FMD response calculated using change from baseline to peak diameter divided by baseline and reported as a percentage value (% FMD). Velocity analysis performed over a minimum of 5 cardiac cycles and averaged, then converted to flow by multiplying by the cross-sectional area of the artery. Vascular measurements were then taken at baseline, 1, 2, 4, 6 and 24 hours post-intervention.

3.2.7.2 LASER DOPPLER IONTOPHORESIS (LDI)

LDI is the gold-standard measurement used as a surrogate marker of microvascular function [337, 608]. It is non-invasive and assesses microvascular response in the forearm after stimulation with a vasodilatory agent [603]. Acetylcholine (Ach) and Sodium nitroprusside (SNP) were used as vasodilators. Acetylcholine is critical in activating endothelium-dependent vasodilation. It binds to the M2 muscarinic receptors of the endothelium surface, leading to the production of nitric oxide (NO) which is responsible for vasodilation as well as vascular smooth muscle relaxation. Sodium nitroprusside is a nitric oxide donor and reacts with sulfhydryl groups *in vivo* to produce NO directly, thus stimulating vasodilation and exerting endothelium-independent control [609]. Measurements were carried out in a temperature-controlled room (22 ± 1 °C) with acclimatised volunteers in a supine position, after calibration of the machine. Two chambers were set 1 cm apart on the alcohol cleansed volar face of the subject's forearm, using double-sided adhesive rings. Chambers were filled with 2.5 ml of 1 % Ach and SNP, respectively, prepared with 0.5 % sodium chloride (Sigma Chemical

Co.). The Ach and SNP were transferred trans-dermally via the anodal and cathodal chambers, iontophoresis was achieved using an electrical current which incremental increase from 0 to 20 µA. Microvascular function was measured by LDI2-IR Laser Doppler imager (Moor Instruments Ltd). The Laser Doppler signal is proportional to the number and velocity of moving blood cells in illuminated, superficial, skin microvessels [610]. The laser beam penetrates the skin and is partially backscattered by moving blood cells, causing, according to the Doppler principle, a frequency shift and generating a signal that is linearly related to red blood cell flow (skin erythrocyte flux), as predicted by theoretical and experimental models [611]. Scans with 5 µA incremental increases in current from 0-20 µA were recorded to measure the elasticity of the small peripheral blood vessels at the lower arm [337]. A median flux against time graph was plotted and the area under the curve calculated to analyse the recorded scans by moorLDI V5.3 Software [612].

3.2.7.3 BLOOD PRESSURE

Systolic and diastolic BP and heart rate were measured in the subject's non-dominant arm, while sitting, with an automatic, digital, blood pressure monitor (Omron M6 Intelli-Sense Comfort; model HEM-72211-E8, Omron Healthcare Co.). Before Measurement volunteers were rested for 30 minutes and the average of triplicate measurements separated by at least 1 min was recorded [613].

3.2.7.4 ANTHROPOMETRIC MEASUREMENT

Before vascular assessments, body composition and weight were measured, using a Body Composition Analyser (Tanita BC-418MA, TANITA, UK Ltd.) by same trained researcher, according to the manufacturer's protocols. Standing height was measured with a stadiometer (Medical scales and measuring system, Seca Lt., UK). Body Mass Index was calculated as weight/height^2 (kg/m^2).

3.2.7.5 ENZYME-LINKED IMMUNOASSAYS (ELISA)

a) ACE activity

Serum angiotensin converting enzyme (ACE) activity was measured by a spectrophotometric method using an ACE Kinetic test kit (Bühlmann Laboratories AG, Schonembuch, Switzerland). Briefly ACE mediates the hydrolysation of the synthetic substrate N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) to an amino acid derivate (furanacryloyl-L-phenylalanine, FAP) and a dipeptide (glycylglycine, GG), the decrease in absorbance during the cleavage reaction is measured [614].

ACE activity was measured using enzymatic assay (ACE Kinetic test kit, Bühlmann Laboratories AG, Switzerland). The substrates, calibrator, controls and the serum specimen were allowed to reach room temperature, high and low controls were prepared by adding 2 ml of deionized H_2O. A plate was prepared with 250 µl substrate and 25 µl calibrator serum sample, then incubated in a Spark®

multimode microplate reader (Tecan Trading AG, Switzerland) for 21 min at 37 °C, after first shaking for 2 min. The kinetic reaction was measured at 3 min intervals from 6 min onwards. The cleavage reaction's kinetic energy was measured as a decrease in the absorbance at 340nm, calculated as follows.

$$ACE\ Activity = \frac{\text{calibrator value} * \text{slope(sample)}}{\text{slope(calibrator)}}$$

b) ACE Concentration

ACE concentration was determined using the method described in Section 2.2.9 for shed ACE, with the following differences. Blood serum replaced HUVEC cells as the tested medium. Serum samples were prepared in a 10-fold dilution by adding Calibrator Diluent RD6-45 (180 µL) to 20 µL of serum.

c) Renin Concentration

The renin concentration in plasma was measured using a quantitative sandwich enzyme immunoassay (Quantikine® ELISA Human Renin Immunoassay, R&D Systems Europe, Ltd., UK) which requires a human renin specific monoclonal antibody pre-coated microplate. Standards and samples were pipetted into wells in the plate; the immobilized antibody binds available renin to form an enzyme-linked monoclonal antibody. A substrate solution, which produces a detectable signal owing to a colour change, was then added. A wash buffer was prepared by mixing a manufacturer supplied concentrate (20 ml) with deionized H_2O up to 500ml. Calibrator diluent RD5P was then diluted at a ratio of 1:5, comprising 10ml calibrator diluent and 40 ml dH_2O. Diluted Calibrator Diluent RD5P (75 µL) was mixed with an equal amount of plasma sample with EDTA anticoagulant. The serial dilution with the human renin standard was also prepared. For reconstitution, 1ml of dH_2O was mixed with the human renin standard (20,000 pg/ml) and was left to rest for 15min prior to use. Again, using a ratio of 1:5, the standard was mixed with calibrator diluent RD5P in 2000, 1000, 500, 250, 125, 62.5, 31.3 and 0 pg/ml, to prepare the serial dilution. High Control, Medium Control and Low Control vials were filled with H_2O (2 ml) and mixed throughout. To each well, 100 µL of assay diluent RD1S was added with a multichannel pipet, prior to adding 50 µL of the prepared standards, control or samples respectively. The plate was then sealed with adhesive strip and incubated for 2 h at room temperature on the orbital microplate shaker (Stuart SSM5 Shaker, Microtitre, Mini, Cole-Parmer, UK) at 500 rpm. After 4 washes with wash buffer (300 µL) and removal of all the liquid, a human renin conjugate (200 µL) was added to each well, wells were again sealed and incubated for a further 2 h at room temperature as previously. After repeating the washing procedure 4 times, 200 µL of substrate solution was added to each well. The plates were re-incubated for 30 minutes at room

temperature while protected from light. Thereafter, a (50 µL) stop solution was added and optical density determined using a Spark ® multimode microplate reader (Tecan Trading AG, Switzerland) at 450nm.

3.2.8 SAMPLE SIZE AND STATISTICAL ANALYSIS

The power calculation was made for the primary clinical outcome measure (FMD of the brachial artery) to determine the minimum number of participants required for the study. The sample size was calculated using the variance of repeated measurements in the control group and on the control data; standard deviation within the participants was 0.35 % (based on previous studies performed in our group). A sample size of 13 subjects yielded 80 % power at $P \leq 0.05$ significance level to determine a significant within-subject difference between treatments of at least 0.3 % of FMD. However, in order to provide power to use the secondary measures and achieve significant within-subject differences, we aimed for 15 participants.

To identify differences in the study outcomes (i.e. markers of CVD risk including LDI and BP) between assessment time points and interventions, a mixed model was used to analyse the effect of covariance response variables based on log likelihood, while a pairwise comparison tool used to analyse the significant differences between variables which are time, intervention and interaction between time and intervention. RAAS enzymes levels and activities in biological samples were analysed by GLM to show a significant effect of covariance variable on the response variables. Then, for comparison we used *post hoc* analysis (Tukey comparison) ($P = 0.05$). All statistical analyses were carried out using the statistical package for social science (SPSS) version 24.

3.3 RESULTS

The sequence of screening, assessment and study visits followed by each participant is presented in Figure 3.2 and the study population's baseline physical characteristics at screening are shown in Table 3.2. All participants were male, healthy and diagnosed with pre- or stage 1 hypertension. The results of statistical analyses of the intervention and control groups over the measurement period shown in Figure 3.1 are shown in Table 3.3. Results are presented for each of the primary and secondary study objectives.

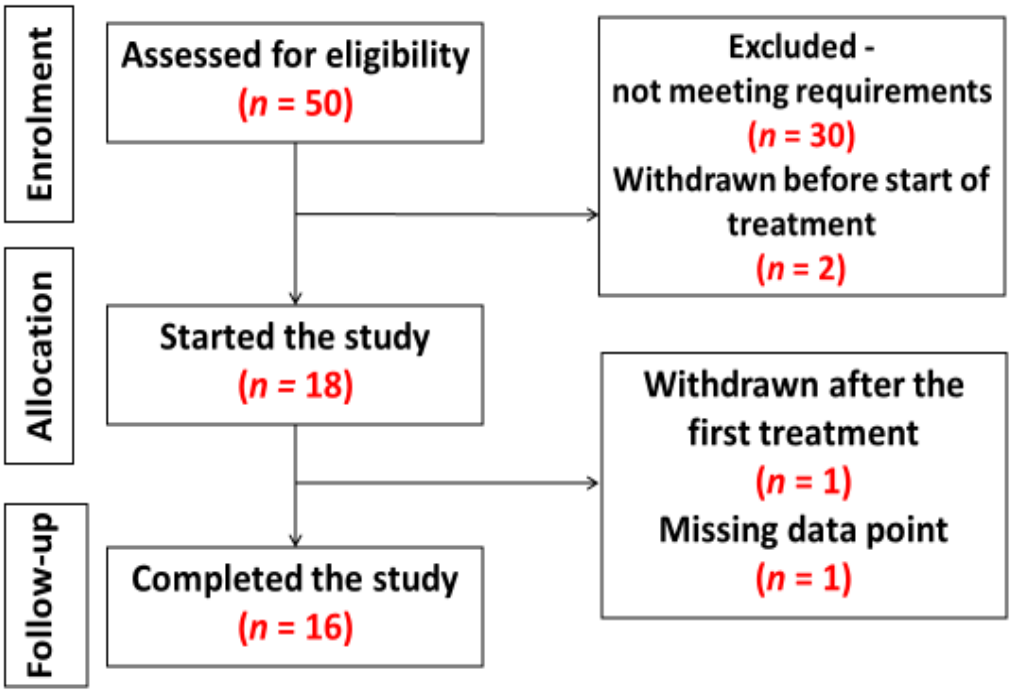


Figure 3.2 Flow chart of study participation

18 participants enrolled, and 16 completed both assessment visits. Reasons for exclusion / failure to complete are shown to the right.

Characteristic	Mean ± SEM	Range
Age (y)	47.3 ± 3.3	22.0 - 68.0
BMI (kg/m^2)	26.4 ± 0.9	18.9 - 33.5
Body Fat (%)	23.2 ± 1.8	8.0 - 34.7
Systolic blood pressure (mm Hg)	132.1 ± 1.7	117.7 - 147.3
Diastolic blood pressure (mm Hg)	80.5 ± 0.9	74.0 - 86.3
Heart rate (bpm)	71.2 ± 2.4	55.3 - 95.3
Plasma glucose (mmol/L)	5.4 ± 0.1	4.5 - 6.5
Plasma total cholesterol (mmol/L)	5.2 ± 0.3	3.0 - 7.0
Plasma triglycerides (mmol/L)	1.6 ± 0.2	0.6 - 4.8

Table 3.2 Baseline characteristics at screening

Characteristics of overnight fasted study participants at screening; (n = 16).

Measure	Figure	Intervention A - oats B – control	Baseline	Time after intervention (Hours)					Probabilities (P)		
				1	2	4	6	24	Treat-ment	Time	Treat-ment × Time
FMD	3.3	A	4.16 ± 0.57	4.37 ± 0.7	4.01 ± 0.67	5.03 ± 0.84	3.42 ± 0.59	4.44 ± 0.38	0.5615	0.1829	0.482
		B	4.67 ± 0.54	3.9 ± 0.86	3.41 ± 0.45	5.48 ± 0.8	4.42 ± 0.8	3.55 ± 0.65			
Ach-iAUC	3.4 (A)	A	538.16 ± 90.36		681.81 ± 182.02	633.43 ± 82.01	564.87 ± 66.25	381.31 ± 61.41	0.8948	<.0001	0.0679
		B	465.03 ± 70.64		463.69 ± 55.36	702.63 ± 30.43	649.67 ± 51.47	314.97 ± 45.54			
SNP-iAUC	3.4 (B)	A	661.12 ± 52.37		709.43 ± 68.43	555.22 ± 85.84	552.12 ± 60.19	622.38 ± 50.57	0.1572	0.7274	0.0074
		B	757.06 ± 43.24		502.88 ± 25.58	623.28 ± 56.97	681.13 ± 63.73	513.72 ± 35.77			
Ach-AUC	3.5 (A)	A	1593.9 ± 198.85		1678.3 ± 140.6	1676.44 ± 233.96	1627.05 ± 182.27	1560.91 ± 161.29	0.9769	0.2647	0.0777
		B	1534.1 ± 144.24		1358.26 ± 116.9	1621.82 ± 167.51	1757.69 ± 140.77	1701.63 ± 190.89			
SNP-AUC	3.5 (B)	A	1897.85 ± 159.41		1693.55 ± 131.09	1406.17 ± 146.66	1795.97 ± 184.83	1889.87 ± 221.77	0.9831	0.133	0.249
		B	2163.44 ± 209.1		1694.75 ± 144.7	1723.88 ± 140.09	1760.05 ± 220.52	1903.8 ± 158.3			
SBP	3.6 (A)	A	125.38 ± 2.27	125.8 ± 2.24	125.72 ± 2.08	124.44 ± 2.28	126.37 ± 1.57	122.37 ± 1.54	0.988	0.0064	0.5801
		B	124.42 ± 2.3	124.93 ± 1.75	123.94 ± 1.83	122.28 ± 1.34	127.07 ± 1.93	123.77 ± 1.84			
DBP	3.6 (B)	A	73.88 ± 1.51	72.75 ± 1.53	73.32 ± 1.45	74 ± 1.65	71.38 ± 1.49	72.47 ± 1.71	0.6927	0.0207	0.9561
		B	74.15 ± 1.33	72.56 ± 1.27	73.45 ± 1.4	74.64 ± 1.55	72.78 ± 1.44	73.57 ± 1.87			
HR	3.6 (D)	A	64.66 ± 2.84	63.94 ± 2.49	62.1 ± 2.39	61.25 ± 2.08	62.69 ± 1.71	64.15 ± 2.61	0.9757	<.0001	0.9139
		B	64.94 ± 3.18	63.69 ± 2.73	61.84 ± 2.19	61.12 ± 2.13	65.42 ± 2.27	64.87 ± 2.64			
Renin level	3.7 (A)	A	763.79 ± 61.94		753.12 ± 55.24		805.19 ± 77.1	805.99 ± 62.54	0.736	0.0107	0.6903
		B	788.38 ± 45.22		733.05 ± 45.62		776.07 ± 52.69	845.44 ± 64.59			
ACE level	3.7 (B)	A	136.41 ± 8.6		136.38 ± 8.55		134.07 ± 4.85	146.5 ± 6.18	0.0711	0.1393	0.652
		B	150.18 ± 5.42		135.37 ± 8.46		131.07 ± 9.32	136.68 ± 7.37			
ACE activity	3.7 (C)	A	31.76 ± 5.08		28.49 ± 4.06		28.32 ± 6.18	29.03 ± 3.15	0.6786	0.6048	0.7347
		B	30.8 ± 3.38		26.93 ± 2.98		23.62 ± 2.78	30.38 ± 3.96			

Table 3.3 Results of Measurements of Cardiovascular Risk Biomarkers

Values are presented as mean measurement ± SEM, for units see the text. Primary outcomes: flow-mediated dilatation (FMD). Secondary outcomes: endothelial-dependent vasodilation induced by Ach and endothelial-independent vasodilation induced by SNP, both measured by LDI and expressed as both area under curve (AUC) and incremental area under curve (iAUC); systolic blood pressure (SBP); diastolic blood pressure (DBP); heart rate (HR); angiotensin converting enzyme (ACE) and renin. Results from ANOVA of treatment, time, and time × treatment interaction, significant probabilities are highlighted ($P < 0.05$ – light pink, $P < 0.01$ – dark pink). *Post hoc* analyses used Tukey's comparison test. For all measures, n = 16.

3.3.1 FLOW-MEDIATED DILATATION

None of the measured changes in % FMD was significant (CI = 95 %) (Table 3.3 above), baseline % FMD measurements were 4.16 with the oat and phenolic acid intervention, compared to 4.67 % in the control. Following the control intervention, mean FMD was observed to fall up to the 2 h measurement, reaching a low point of 3.41 %, before increasing to a peak at the 4 h measurements and then falling back to 3.55 %, below the baseline, after 24 h. The profile (Figure 3.3) was similar following the oat and phenolic acid intervention, although the reduction over the first 2 h, to 4.37 %, was less than the control (0.1 compared to 1.56 %); measurement at 24 h was the peak, also at 4 h was 5.03 %, however the lowest FMD was measured after 6 h at 3.42 %, and the final FMD rose again at 24 h to 4.44 %. FMD comparisons at 1 and 2 h were improved by 0.21 % and dropped by 0.15 % respectively, compared to the baseline level, in the oat group, however, dropped by 0.77 % and 1.26 % compared to the baseline level, in the control group. At 4 h for both groups, FMD was 0.87 % above the baseline for the oat compared to only 0.81 % in the control group.

Differences between the means of the oat and control treatments, the treatments over time and the interaction between them were not significant (Figure 3.3); treatment (P = 0.5615), time (P = 0.1829) or the time × treatment interaction (P = 0.482).

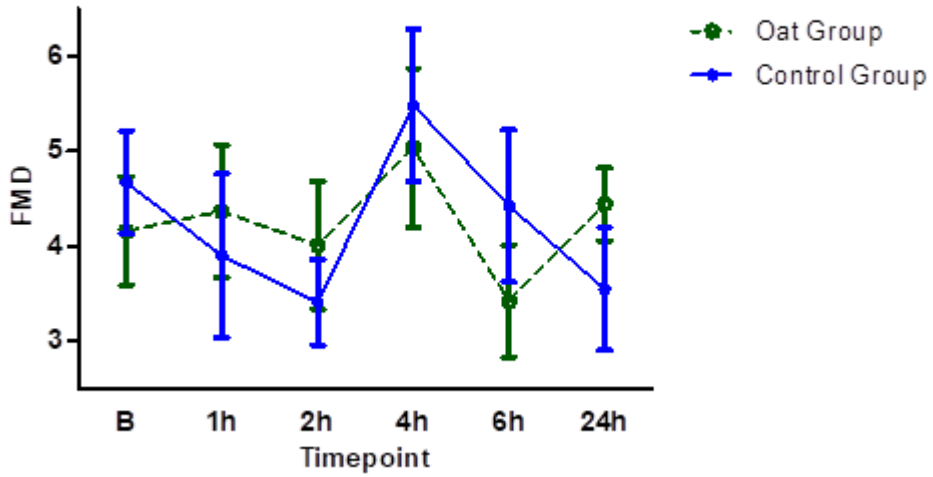


Figure 3.3 Changes in FMD after consumption of phenol-rich, wholegrain Oats

Oat Group consumed phenol-rich, wholegrain oats containing 45.0 mg of phenolic acids and 5.0 mg avenanthramides, compared to the Control Group. Values are means ± SEMs from Table 3.3 FMD: flow-mediated dilatation. GLM analysis, n=16, followed by Tukey *post hoc* test (P > 0.05).

3.3.2 LASER DOPPLER IONTOPHORESIS

Figures 4 and 5 present the results from Table 3, showing LDI vasodilation measurements over time expressed as area under curve (AUC) and incremental area under curve (iAUC), using Ach and SNP as vasodilators. Differences in vasodilatory responses due to altered NO production, endothelial-dependent or -independent, can be distinguished by LDI following doses of Ach and SNP, respectively, and provide evidence of the receptor sites on which oat polyphenols act.

Results for LDI cannot be considered conclusive, since this is a secondary outcome of the study and the results for FMD, the primary outcome, were not significant; the study design only ensured sufficient power to reliably analyse the primary outcome. iAUC of endothelium-independent vasodilation, facilitated by SNP, when analysed in a linear mixed model, with *post hoc* Tukey's comparison (Table 3.3) showed a putatively highly significant ($P = 0.0074$) interaction between treatment × time, with a peak in vasodilation at 2 h in the oat intervention group (709.43 compared to 502.88 PU/h^{-1} in the control) (Figure 3.4(B)) but was not significant for AUC. In contrast, analysis of endothelium-dependent vasodilation, with Ach as the vasodilator, was non-significant for the same interaction using both AUC and iAUC ($P = 0.0777$ and $P = 0.0679$ respectively) (Table 3.3, Figures 3.4 (A) and 3.5 (A)). There is also an indication from iAUC that acute consumption of avenanthramide and phenolic oats may lead to an improvement in endothelium-dependent vasodilation over time ($P < 0.0001$), but no other endothelial dependent vasodilation results reflected this finding nor were any significant for phenolic oat interventions only.

Graphic results, Figures 3.4 and 3.5, showed that for the control group the profiles are similar for endothelium-dependent and -independent vasodilatory changes using both iAUC, and AUC. The pattern is, however variable in the oat intervention group, which shows comparable profiles, a rise and then gradual decline in vasodilation, for endothelium-dependent vasodilation using AUC and iAUC. A different profile emerged in endothelium-independent vasodilation, when the initial rise followed by decline in vasodilation was followed by a final recovery towards the baseline levels in both cases, suggesting that the effects of polyphenol treatment on endothelium-independent vasodilation may have a short duration.

Our results indicate, therefore, that maintaining local levels of NO as a mechanism by which phenolic-rich foods may reduce the risk of endothelial dysfunction and hence, atherosclerosis, merits further investigation.

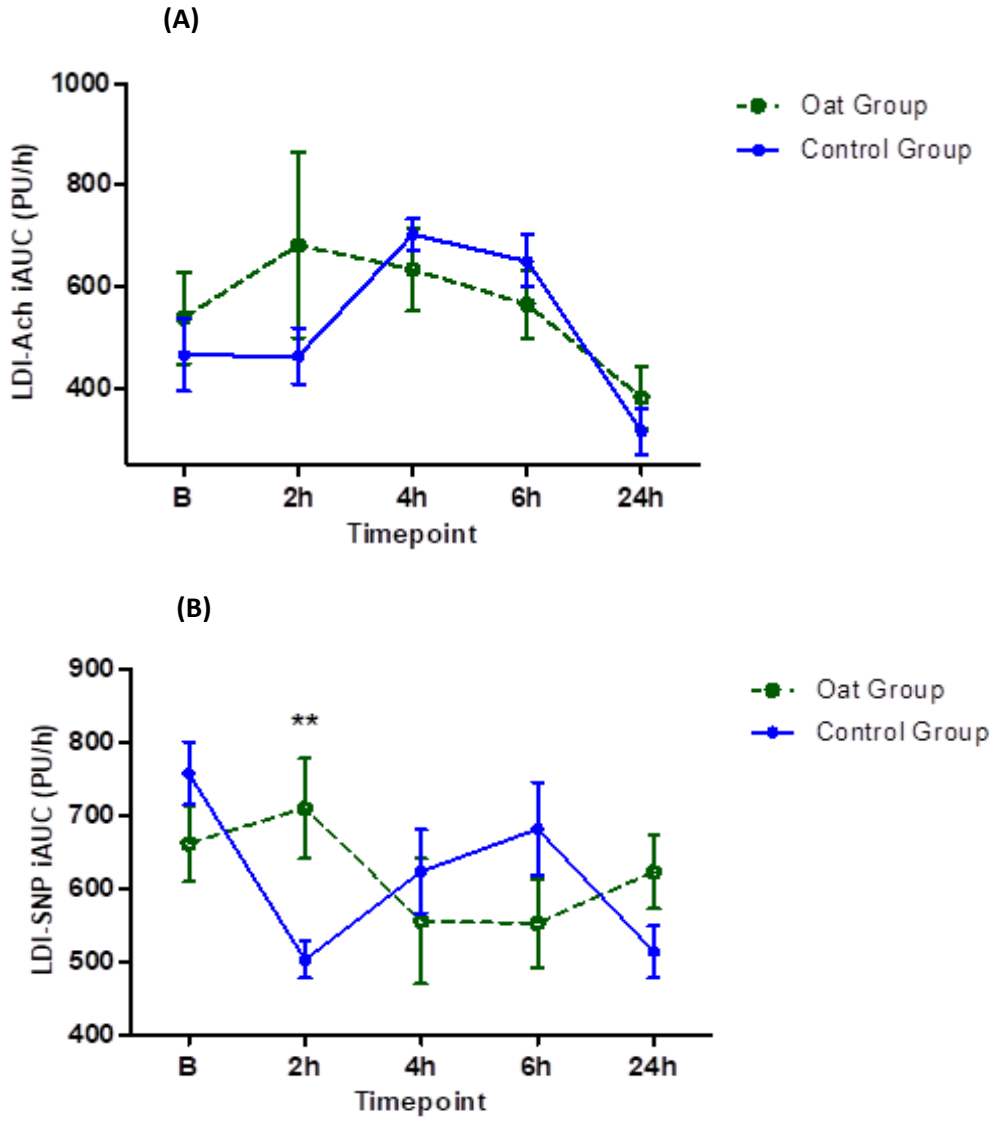


Figure 3.4 Effects of oat treatments on: (A) endothelium-dependent vasodilation (Ach) and (B) endothelium-independent vasodilation (SNP), expressed as iAUC.

Effects of phenolic-rich, wholegrain oat consumption, compared to a control over 24 h in pre- or stage 1 hypertensive men, represented by incremental area under the curve (iAUC) (perfusion units per hour, PU/h-1), on: (A) endothelium-dependent vasodilation (Ach) and (B) endothelium-independent vasodilation (SNP). Mean measurements are shown from Table 3.3 at baseline (B) and 2, 4, 6 and 24 h post intake are shown; vertical bars – standard error. Significant differences are highlighted - **. A mixed model analysis, n = 16, followed by Tukey post hoc test (P > 0.05).

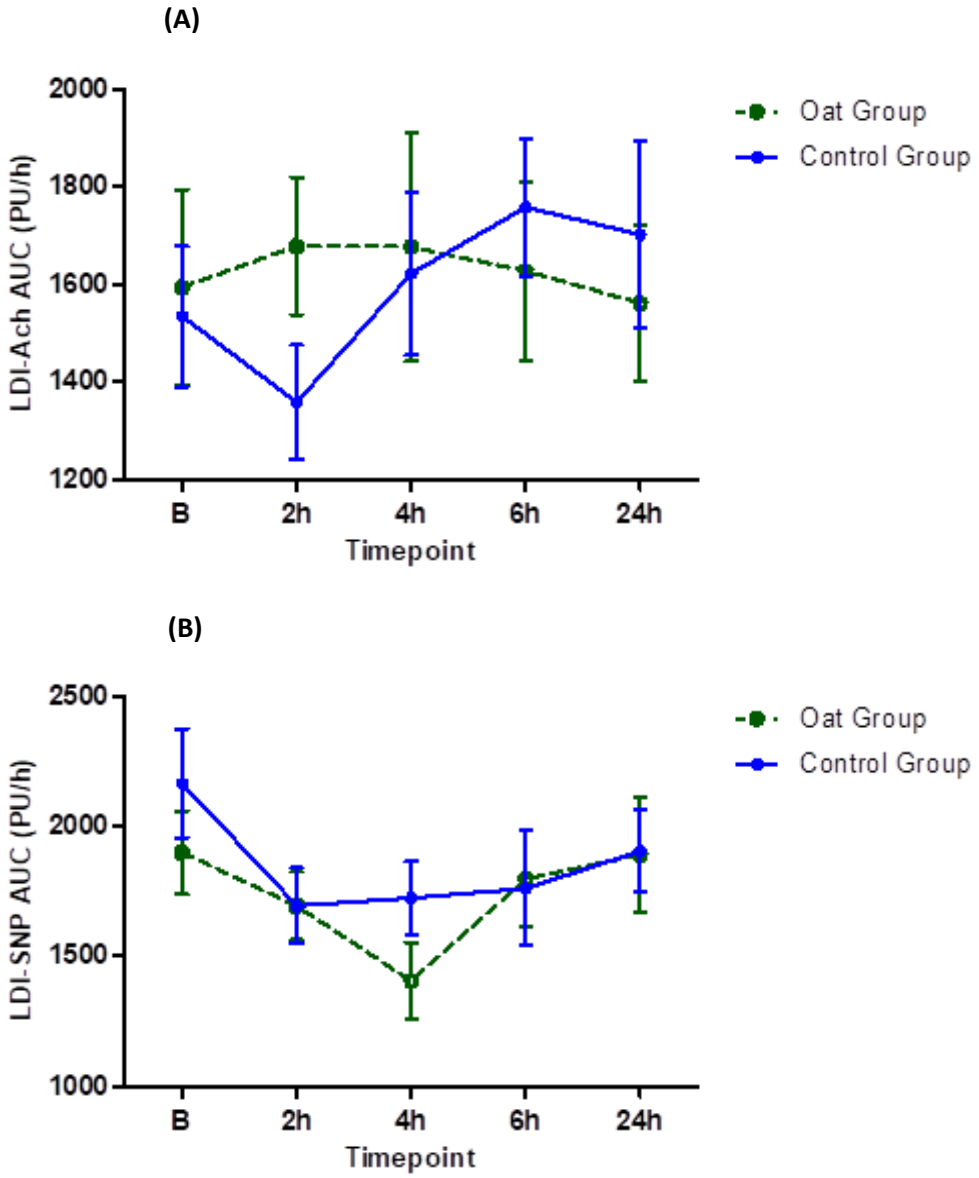


Figure 3.5 Effects of oat treatments on: (A) endothelium-dependent vasodilation and (B) endothelium-independent vasodilation, expressed as AUC.

Effects of phenolic-rich, wholegrain oat consumption, compared to a control over 24 h in pre- or stage 1 hypertensive men, expressed as area under the curve (AUC) (perfusion units per hour, PU/h^{-1}), on: (A) endothelium-dependent vasodilation (Ach) and (B) endothelium-independent vasodilation (SNP). Mean measurements are shown from Table 3.3 at baseline (B) and 2, 4, 6 and 24 h post intake are shown; vertical bars – standard error. A mixed model analysis, n = 16 followed by *Tukey post hoc* test ($P > 0.05$).

3.3.3 BLOOD PRESSURE, PULSE PRESSURE AND HEART RATE

Peripheral SBP, DBP, pulse pressure (PP) and heart rate (HR), all failed to show any significant differences between acute consumption of avenanthramide and phenolic acids-rich oats and the energy matched control treatments at any measured time-point (1, 2, 4, 6 and 24 h) (Figure 3.6), when analysed with p*ost hoc* Tukey's multiple comparisons test. Although SBP and HR showed highly significant differences ($P = 0.0064$ and $P < 0.001$ respectively) in mean measurements over the measurement period (Time, Table 3.3) compared to the baseline measurements and DBP also showed significant differences ($P = 0.0207$), these are secondary outcomes and therefore indicative rather than conclusive results. Of these secondary objectives, only pulse rate did not change significantly over time. Unlike the vasodilation results, these indicators did not show any significant differences in the interaction between treatment and time, further evidence of a low level of influence from the intervention.

Despite the lack of significance, the results do show similar change-profiles for SBP, DBP and HR (Figure 3.6), with all three indicators showing lower baseline measurements in the oat treatment group, followed by sharp increases 1 h into the measurement period, at which point they all slightly exceeded the control group (SBP 125.8 mm Hg compared to 124.93, DBP 72.75 mm Hg compared to 72.56 and HR 63.94 beats/min^{-1} compared to 63.69), followed by a relatively steep drop, to below the control group levels by 2 h. From 4 – 24 h changes in both treatment and control are equivalent, with the oat treatment showing measured consistently lower than the control, graph profiles, however, differ slightly between the three indicators. The profile of changes in pulse pressure (Figure 3.6 (C)) is similar, differing in, however, the absence of the same sharp, control-exceeding peak at 2 h in the oat treatment group.

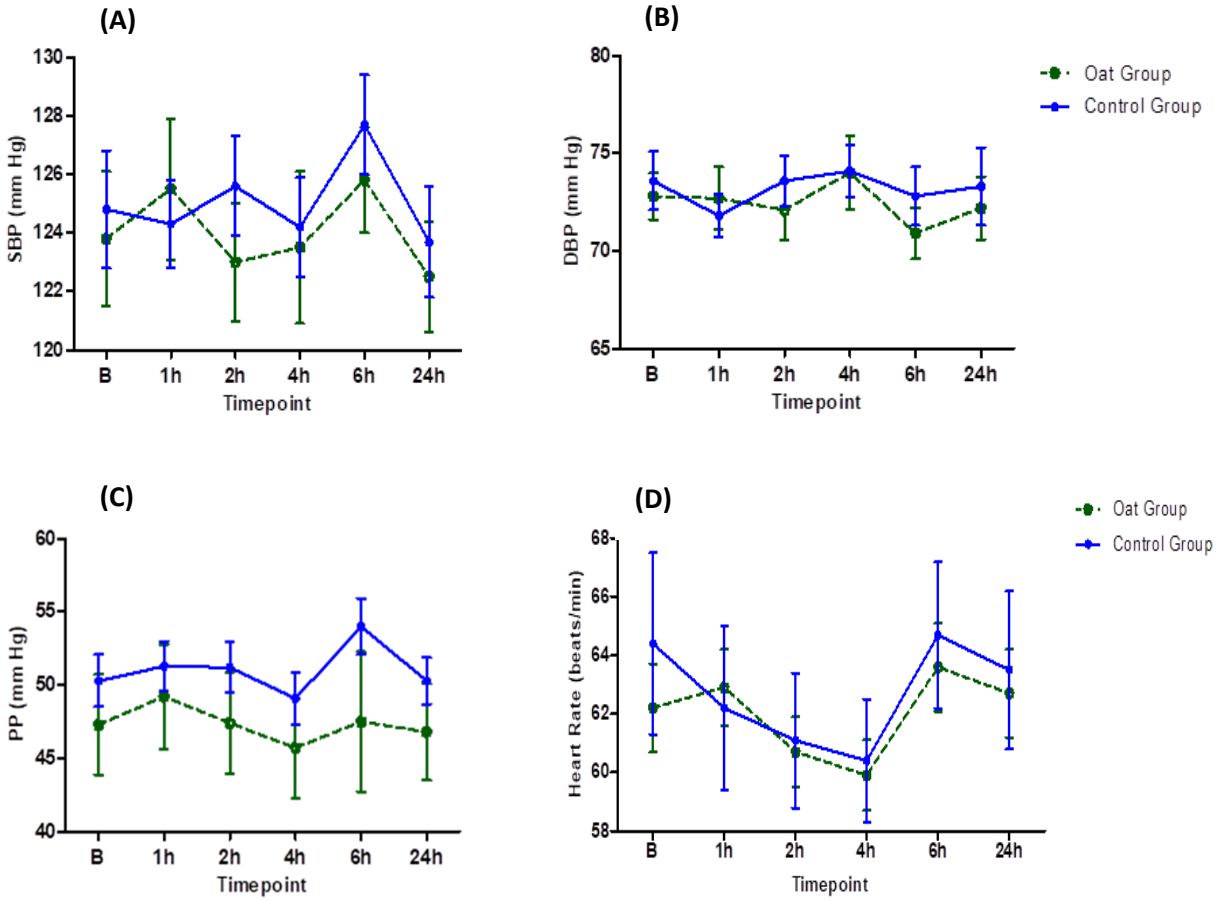


Figure 3.6 Effects of consuming phenolic-rich, wholegrain oats, on: (A) systolic blood pressure (SBP), (B) diastolic blood pressure (DBP), (C) Pulse pressure and (D) Heart rates

Effects of phenolic-rich, wholegrain oat consumption, compared to a control over 24 h in pre- or stage 1 hypertensive men, on:
(A) - systolic blood pressure (SBP); (B) - diastolic blood pressure (DBP); (C) - pulse pressure (PP) and
(D) heart rate.
Mean measurements are shown from Table 3.3 at baseline (B) and 1, 2, 4, 6 and 24 h post intake; vertical bars – standard error. A mixed model analysis, n = 16, followed by Tukey *post hoc* test ($P > 0.05$)

3.3.4 ANGIOTENSIN CONVERTING ENZYME AND RENIN ENZYME INHIBITION

In a GLM then *post hoc* analysis (Tukey comparison) (Table 3.3), no significant differences were observed in ACE activity in blood serum, or renin levels in blood plasma, between the intervention groups measured, at any measured time-point compared with the baseline measurements, or in the interaction between them. The oat treatment and control groups did, however, show close to significant levels (P = 0.0711) in ACE levels, with greater inhibition following oat consumption possibly suggesting a beneficial effect from treatment according to this secondary measure.

In the oat treatment group, inhibition of renin level and ACE activity were highest after 2 h (Figure 3.7 (A) and (C)), whereas ACE levels were lowest after 6 h (Figure 3.7 (B)). Levels were 805.19 pg/ mL^{-1}, 28.32 U and 134.07 ng/mL^{-1} respectively. In all cases there was a later recovery in enzyme levels, suggesting the effects of the treatments were wearing off, which for renin and ACE levels, eventually (by 24 h) exceeded the baseline measurements. The control groups also showed similar initial enzyme inhibition followed by recovery, with greatest inhibition at 2 h for renin, compared to 6 h for ACE in both the levels and activity measurements. Although no results were statistically significant, there are indications from our results that the consumption of the treatment, irrespective of the nutritional differences between the oats and the control, within the constraints of this study, may lead to an initial, RAAS enzyme inhibition, followed by recovery to, or in excess of, the baseline measurements.

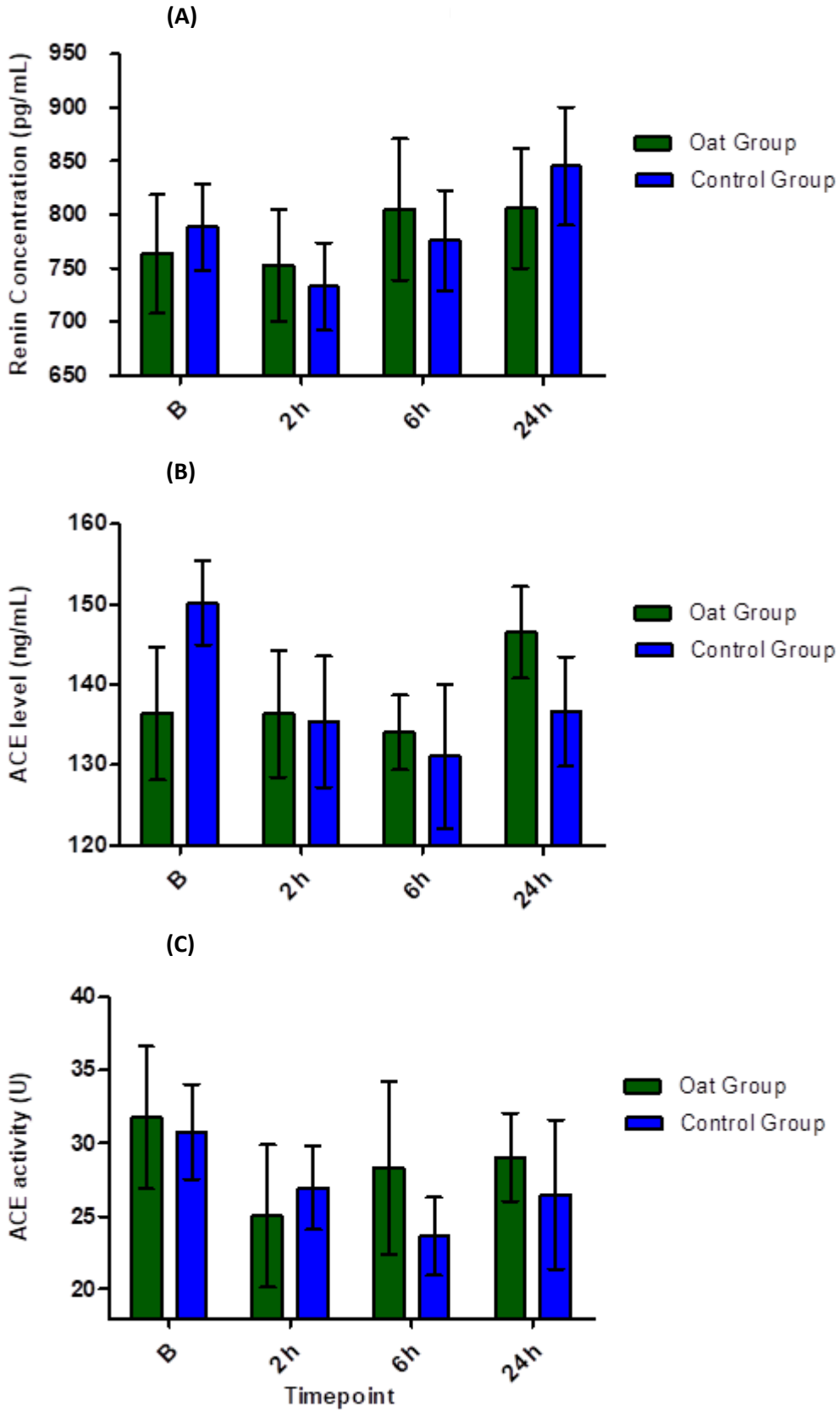


Figure 3.7 Effects of consuming phenolic-rich, wholegrain oats on: (A) Renin concentration; (B) ACE levels and (C) ACE activity

Effects of phenolic-rich, wholegrain oat consumption, compared to a control over 24 h in pre- or stage 1 hypertensive men, on: (A) Renin levels (pg/ml^{-1}) in plasma; (B) ACE level (ng/ml^{-1}) and (C) ACE activity (U) in serum. Mean measurements are shown from Table 3.3 at baseline (B) and 2, 6 and 24 h post intake - solid bars; thin bars - standard error. GLM, n=16, followed by Tukey *post hoc* test (P > 0.05).

3.4 DISCUSSION

This study aimed to test the hypothesis that a single acute intake of oats would reduce BP and improve endothelial function, in men with pre- or stage 1 hypertension, and that these physiological changes would be measurable in effects on the RAAS, specifically ACE and renin inhibition. Our data indicate that at the intake amount used there was no significant change in mean % FMD or BP following oat consumption, relative to a control, but an increase in endothelium-independent vasodilation at early time points, as assessed by LDI with iontophoresis, was induced. Although there are no similar clinical trials with oats to directly compare with our data, i.e. ones that isolate the impact of the phenolic acids from other potential bioactives in oats, clinical vascular data exist for the impact of other phenolic-containing foods and beverages [335, 575-579]. For example, 400 mg pure chlorogenic acid intake has been shown to decrease systolic and DBP, although did not influence nitric oxide status or endothelial function acutely [255]. Our vasodilation results, while not showing significant responses to the intervention, agree broadly with a randomised, placebo-controlled trial indicating that the moderate intake of champagne acutely improves endothelium-independent vasodilation at 4 and 8 h post consumption [343], particularly with respect to endothelium-independent blood flow 2 h following intake. Coffee intake has also been shown to improve endothelium-dependent brachial artery (FMD) at 1 and 5 h following intake in acute trials, which was paralleled by the appearance of hydroxycinnamate such as chlorogenic acid metabolites in plasma [577]. The impact of oat polyphenol intake on the vascular system, however, appears to be more subtle that observed for the coffee study, which administered higher doses of phenolic acids compared to our oat intervention (310 mg compared to 50 mg for oat). This may be why our results failed to induce significant responses. Similarly, the relatively low oat intake in our study failed to induce the magnitude or statistical reliability of FMD increase and BP reductions observed for flavonoid-containing interventions such as cocoa [498, 581], berry [49, 335, 580] and green or black tea [351, 584].

Our study is broadly in agreement with other clinical trials which suggest the consumption of avenanthramide-rich oats may mediate vasodilation [487, 601], although our data show weaker associations with vascular outcomes and were not reliably statistically significant. This is likely due to differences in study design, particularly that of the intake dose used in the various trials and the small sample size. For example, oat bread intake has been shown to not affect FMD over a 4 week period, although it did induce a significant increase in baseline brachial artery diameters and post-ischemia diameters [602], changes that were only observable as trends in our results. Notably, this study used an intake of 30 g β-glucan per day, compared to our 5.6 mg, further evidence that our interventions were not sufficient to elicit significant results. Other studies have also, however, failed to demonstrate chronic increases in FMD, with whole grain oat or wheat cereals having no effect on brachial artery vasodilatation when consumed with a high fat diet. Oat, attenuated reduction of augmentation index

(AIx), one form of pulse wave analysis (PWA) following the high fat meal [487, 615], although similarly to n-3 PUFAs, oat consumption may attenuate declines in brachial artery flow response induced by a high fat intake [603]. In addition, consumption of green oat extracts at 1500 mg, is reported to improved vasodilator function of systemic and cerebral arteries [53]. Our results are also similar to a study of blueberry ingestion which, although dosing with 300 mg, an intake much higher than ours, nevertheless concluded no significant differences took place in peripheral arterial function [616].

Our observations regarding a lack of influence of oat phenolic intake on BP were reflected in a lack of influence on the RAAS, however, our results were non-significant and, therefore, only indicative. RAAS inhibition is thought to be a physiological target for the prevention of high BP and thus CVD [552, 617], for example, *in silico* hydrolysates of oats and their *in vitro* bioassay showed that oat protein isolates did aid in renin and ACE inhibition [374, 618]. However, these studies used whole grain oats and do not directly compare the effects associated with avenanthramide and phenolic acid metabolites; the *in vitro* responses to oat protein and inhibition of ACE enzymes, renin, and dipeptidyl peptidase-IV they found were, however, significant. It is easier to measure enzyme inhibition *in vitro* rather than *in vivo*, as many different variables, factors and environmental conditions can be controlled *in vitro*. Such variables include bioactive peptide degradation by gastrointestinal digestion, the maintenance of bioavailability for exerting a favourable physiological effect, predicted solubility, toxicity and allergenicity. Furthermore, only blood enzyme activity can be measured *in vivo* and not cellular enzyme activity, which limits our ability, currently, to detect potential cellular level benefits of avenanthramides. Another study of flavonoids, notably in tea, found significant inhibition of ACE activity which led to improvements in vascular health [351]. In addition, the plant polyphenol quercetin was found to be associated with a reduction in BP, however, the reduction was noted independently of changes in ACE activity [298]. Our study also found no significant change in ACE activity, however, our results differ from these findings with quercetin, in that our results appeared, within their limitations, to show no BP reduction. While several studies have indicated an inverse relationship between dietary fibre/whole grain content and hypertension, these trials utilised high dietary intake of fibre. However, the reported antihypertensive effects from whole oats or β-glucan ingested in high concentrations or volumes are not consistent, since in another study they were found to have conspicuously antihypertensive effects in individuals with two risk factors obesity and hypertension [619].

Acute BP reductions after polyphenol intake are widely recognised, however, some acute trials have reported no significant effects of their intake on BP. Our data ostensibly agree with studies of flavonoid-rich acai and cranberry, which both also failed to measure significant effects on blood pressure [335, 592]. A systematic review of the effects of oat consumption on BP, found significant reductions were only identified in 4 of the 20 studies considered [40]. Other studies have indicated potential decreases

in cardiovascular risk to individuals consuming whole grain oats through reduced LDL cholesterol while also still failing to detect significant reductions in blood pressure [620-622]. In a randomised, controlled trial involving 16 participants in the age group 51–69 y who were given orange juice, it was found that in spite of high serum levels of plasma flavanones and phenolics (15.20 ± 2.15 μmol/L^{-1}) no changes in cardiac risk biomarkers including BP could be detected [623].

Therefore, using a robust design, our study revealed that a single moderate intake of avenanthramide and phenolic acid enriched oats may influence mean % FMD beneficially, because there was a medically relevant magnitude of effect relative to the control, although these changes failed to achieve statistical significance. There was no significant change in mean BP following the oat intervention, relative to the control, but an increase in endothelium-independent vasodilation, again not detected as significant in the models we used, was induced at early time points.

The use of an all-male sample reduces the likelihood of confounding factors between volunteers in this acute trial and also makes the results more comparable to other studies. However, the short-term intervention and the lack of investigation of differences in rates of metabolism and variations in responses to measurements and sample collections between volunteers contributed to the limitations of the trial outcomes. Furthermore, the trial was uninformative with respect to the long-term effects that oats consumption might have on CVD biomarkers, and our knowledge, considering also the limited availability of published studies, remains, therefore, incomplete. A further study was, therefore, planned and conducted with a chronic design and a larger sample size, to analyse the long-term effect of oats consumption in a similar at-risk group for cardiovascular disease, which is described in Chapter 4.

Chapter: 4 Investigation of the Vascular Effects of Chronic Oat Intake on Adults with Pre- and Stage 1 Hypertension

4.1 INTRODUCTION

This chapter considers the vasoactive potential of a chronic intake of medium and high dosage avenanthramide and phenolic acid containing oats on pre- and stage 1 hypertensive adults. Of the numerous published epidemiological and human intervention studies concerning the cardiovascular effects of oats, the overwhelming focus to date has been on the potential benefits of increased whole grain or whole grain fibre consumption and on diets or interventions incorporating β-glucan. This approach has frequently failed to account for the roles and contributions of the various oat constituents known to be bioactive, which include, vitamins and minerals, polyphenols in general and specifically small phenolic compounds. Since chronic intervention studies have usually patently failed to differentiate the specific effects of the polyphenolic content from other bioactive constituents, despite the amassed body of evidence from such studies, our understanding of both the most efficacious dietary ingredients and by implication the pathways of their action, is far from complete. Most studies investigate chronic interventions, emphasizing a dietary rather than medicinal view of the potential benefits of polyphenols, and we describe some characteristic and pertinent chronic intervention studies below. We also describe the scope of phenolic compounds which are contained in oats. There is some limited *in vitro* confirmation that avenanthramides [436] and phenolic acids (PA) [593] may be capable of influencing CVD and related risk factors and by investigating these, the most prominent phenolic components of oats, at different but dietary realistic intervention levels, in a chronic intervention on subjects with physiological characteristics that are recognised to be likely to be susceptible to such intervention, i.e. pre-hypertensive adults, this set of assays will make a substantial and novel contribution to filling some important gaps in our understanding

Diet is known to be capable of influencing an individual's blood pressure and represents one of the most important modifiable risk factors. For example, epidemiological studies have shown that CVD risk is elevated by the intake of high levels of fat, particularly trans and saturated fat [39, 624-626], and high levels of dietary sodium intake [627-629]. Conversely, CVD risk factors such as hypertension have been shown to be ameliorated by high dietary intake of vegetables, fruits and dietary fibres [13, 36, 630, 631]. Whilst various macro- and micro-nutrient constituents of these foods may impact on CVD development and related risk factors, such as blood pressure [632], recently plant polyphenols including flavonoids, and phenolic acid, present at high concentrations in fruits, vegetables and whole grains have been shown to impact beneficially on blood pressure and endothelial function [342, 633, 634]. Diets rich in

flavonoids have been shown to improve vascular function [73, 325, 635, 636] and successfully lower BP in mildly hypertensive or normal BP subjects [73, 637, 638]. Specifically, flavanol intake is well reported to decrease blood pressure in patients with hypertension [286], whereas the intake of the flavanone hesperidin decreases DBP in healthy overweight men [639]. Furthermore, polyphenol intake has also been observed to reduce low density lipoprotein (LDL) cholesterol levels, another well-established marker of CVD risk [640] and an improvement in the blood lipid profile in patients with hyperlipidaemia [641]. Coffee intake, which has similar small phenolics to those found in whole grain oats, has been shown to improve vascular function in healthy men [577, 586].

Whole grain oats are a rich source of phenolic acids, in particular ferulic acid p-coumaric acid, 4-hydroxybenzoic acid, syringic acid and sinapic acid [595, 642], in addition to vitamins, minerals, phytosterols, unsaturated fatty acids and lignans [565, 643, 644]. A number of constituents have been suggested to contribute to the cardiovascular health benefits of oat intake, although the majority have been attributed to their fibre content [645-647]. Such a conclusion is reasonable in light of data indicating that intake of soluble fibre (β-glucan) from oats or oatmeal significantly decreases blood cholesterol levels and blood glucose levels [647-651]. β-glucan is the major constituent of the dietary fibre (DF) fraction of oats, with lower amounts of arabinoxylan (AX) and only traces of other cell wall polysaccharides, such as cellulose and glucomannan [652]. Although β-glucan is the dominant DF component in whole grain, AX is of interest as it binds over half of the phenolic acids found in the grain [595]. The remainder are present (in soluble form) either as free acids or conjugates (such as steryl ferulates), along with other potential bioactive components such as avenanthramides (N-cinnamoylanthranilate alkaloids), alkylresorcinols (phenolic lipids), sterols, tocopherols and tocotrienols tocols [595]. As in all cereals, these components are concentrated in the outer layers of the grain (aleurone, pericarp and testa, Figure 1.10) and hence are recovered in the “bran” fraction on milling. Thus the impact of whole grain intake on human cardiovascular health is highly likely to be made up of the combined effects of both fibre and phenolic acids found within the whole grain [645].

Several epidemiological studies have suggested that an increased intake of whole grain products is associated with a reduction in hypertension, type 2 diabetes, and chronic heart disease (CHD) [40, 653-655]. Oats in particular have been shown to have a positive impact on CVD health status, reducing low-density lipoprotein (LDL) cholesterol, SBP and DBP and improving vasodilation and blood flow [53, 547]. It has also been speculated that their intake may reduce the necessity for anti-hypertensive drugs [650]. Whole grain wheat and oat intervention for 12 weeks significantly reduced SBP and pulse pressure (6 and 3 mm Hg respectively), when compared with refined cereal intake [597]. The intake of oats has been inversely associated with coronary heart disease, CVD and stroke by 21, 16 and 11 %, respectively [654, 656]. Increases in % FMD by 1.80 ± 0.50% have been reported in a trial using wild green oat

extract, which contains high levels of phenolic acids and other potential bioactive substances such as saponins, vitexins and isovitexin [53]. Whole grain has been shown to induce significant reductions in both SBP and DBP, in slightly hypercholesterolemic volunteers [657] and hypercholesterolemic men [658], using intervention levels and chronic trial periods similar to those used in this trial. Whole grain oats, in studies of mildly hypertensive subjects found a significant 6 mm Hg reduction in SBP after a 6-week intervention [601] and of 7.5 mm Hg SBP and 5.5 DBP [598, 659].

The RAAS is an important system controlling BP homeostasis, and cardiovascular and renal function in humans and its critical role in probably all amelioration of CVD has been increasingly recognised in recent years [660]. While clinical trials using oats show mixed results with respect to RAAS effects, many *in vitro* studies show strong inhibitory effects of oat polyphenols on RAAS, particularly ACE inhibition and/or renin expression [374, 661]. Wheat inhibited ACE activity *in vitro* [662] and has been shown to strongly inhibit ACE levels in blood plasma [663]. Largescale studies have shown that increased urinary sodium and potassium excretion, as proxies for dietary intake, are associated with CVD and other health risks [664, 665], a 12 week study of the effects of an oat diet on 24h BP in hypertensive men found that excretions remained unchanged following intervention [666]. However, a 5 week whole-grain diet study which reduced SBP and DBP reported no effects on in urinary excretions of minerals related to BP [657]. A study using barley, showed higher decreases in urinary salt excretions compared to different whole grains in hyperlipidaemic subjects [658].

However, studies have not consistently found improvements in cardiovascular health indicators. A meta-review of oat-based chronic studies indicated that no significant changes in a range of CVD risk markers [40] was a common finding. In a study of oat and wheat based whole grain interventions no changes in FMD were found in hypercholesterolemic patients [667]. A larger study of 315 overweight individuals given whole grain oats for four months at different dosages did report small improvements in endothelial function but did not find any significant differences between interventions [668]. A whole grain intervention study found differences in both dependent and independent vasodilation, but none that was significant [669]. It has also been found that ambulatory 24-h BP following oat intake for 12 weeks was not significantly changed in a study of hypertensive men [666]. Other studies which have concluded that whole grain diet led to no significant difference in plasma lipid profiles [668, 670] and in a review of 21 randomised controlled trials where the impacts of oat intake were being studied, only 13 studies showed significant reduction in total cholesterol (TC) and only 14 significant reductions in LDL [656].

This study aims to assess the chronic effects (i.e. after 4 weeks) of consuming whole grain oats, with particular consideration for the levels of polyphenols (avenanthramide and phenolic acids) they contain, on microvascular function and 24 h blood pressure. It also examines a potential mechanism for

observed physiological changes by measuring the level and activity of angiotensin converting enzyme (ACE) and renin.

4.2 MATERIALS AND METHODS

4.2.1 SELECTION OF SUBJECTS

Potential volunteers were recruited using the Hugh Sinclair Unit of Human Nutrition database and local advertisements, local media, social media websites, through poster/leaflet advertisements in local Hospitals and through mail out via local GP practices for the latter two recruitment strategies, NHS ethical approval was done. Interested volunteers were given a participant information sheet (Appendix 6) detailing the background of the study and what was expected of them. Volunteers still interested in participation were asked to fill in a health and lifestyle questionnaire (Appendix 7) to verify that they met the broad inclusion/exclusion criteria, for example age, dietary allergies and medication intake, as outlined below.

Volunteers eligible for inclusion, measured as described below, comprised: males, females taking the contraceptive pill or on hormone replacement therapy (HRT), 25 to 75 years old, above average blood pressure (i.e. systolic 120-159 mmHg and diastolic 75-99 mmHg). Exclusion Criteria were: abnormal biochemical/haematological results assessed at the health screening, hypertension (i.e. SBP/DBP ≥ 160/100 mm Hg), BMI >35, current smoker or ex-smoker ceasing <3 months ago, past or existing medical history of vascular disease, diabetes, hepatic, renal, haematological, neurological, thyroidal disease or cancer, prescribed or taking lipid lowering, anti-hypertensive, vasoactive (e.g. Viagra), anti-inflammatory, antibiotic or antidepressant medication, allergies to whole grains, dairy and lactose intolerance, parallel participation in another research project, having the flu vaccination or antibiotics within 3 months of trial start, chronic constipation, diarrhoea or other chronic gastrointestinal complaint (e.g. irritable bowel syndrome), on a weight reduction regime or taking food, probiotic or prebiotic supplements or laxatives within 3 months of the trial start, performing high levels of physical activity (i.e. ≥ 150min aerobic exercise/week), consumption of alcohol ≥ 21 units/week for men and ≥ 15 units/week for women, females who were breast-feeding, or who may be pregnant, lactating or, if of reproductive age, are not using a reliable form of contraception (including abstinence).

Volunteers meeting the broad criteria attended a short screening visit, after having fasted overnight (i.e. no intake of food or drink for 10h before the visit, except water), where the study was explained in detail, including gastrointestinal questionnaires, 3-day food diaries, the intake of the study intervention materials, the collection of urine sample and dietary & exercise restrictions. A demonstration of the vascular measurements was given. Volunteers were encouraged to ask any remaining questions. Anthropometric measures, blood pressure measurements and 10 ml of blood were collected. Blood

samples were analysed for biochemical and haematological markers partly on site at the University of Reading and partly by the pathology department of the Royal Berkshire Hospital (Reading, UK). Based on screening results, as detailed below, 28 healthy volunteers were invited to participate in the study. All volunteers provided written, informed consent before commencing the study (Appendix 8), while all further aspects of the study were discussed in detail.

4.2.2 STUDY DESIGN

The study was approved by the University of Reading Research Ethics Committee as NCT02847312 and registered at www.clinicaltrials.gov ID 211656 and REC reference 16/LO/1542. The proposed study was a single-centre, three-arm, double-blinded, placebo-controlled randomised crossover intervention. Volunteers were required to attend the Hugh Sinclair Unit of Human Nutrition on six occasions (Figure 4.1) during which they either consumed different doses of the intervention foods, or an energy matched control intervention, selected in random order.

The purpose of the trial was to test the chronic effects (i.e. after 4 weeks) on cardiovascular risk markers and gut health of consuming a daily β-glucan matched breakfast, and afternoon snack that were moderate or high in oat avenanthramide and phenolic acids (hereafter "phenolic oats"). The study intervention materials and quantities used were as detailed in Table 4.1, which includes their nutritional content. Each intervention period was separated by a washout of 4 weeks.

The primary expected outcome was flow-mediated dilatation of the brachial artery (FMD). Secondary outcome measures were microvascular endothelial function (measured by Laser Doppler Iontophoresis), activity and level of the renin angiotensin system, 3-day food diaries and 24 h minerals excretions, which all markers associated with CVD risk.

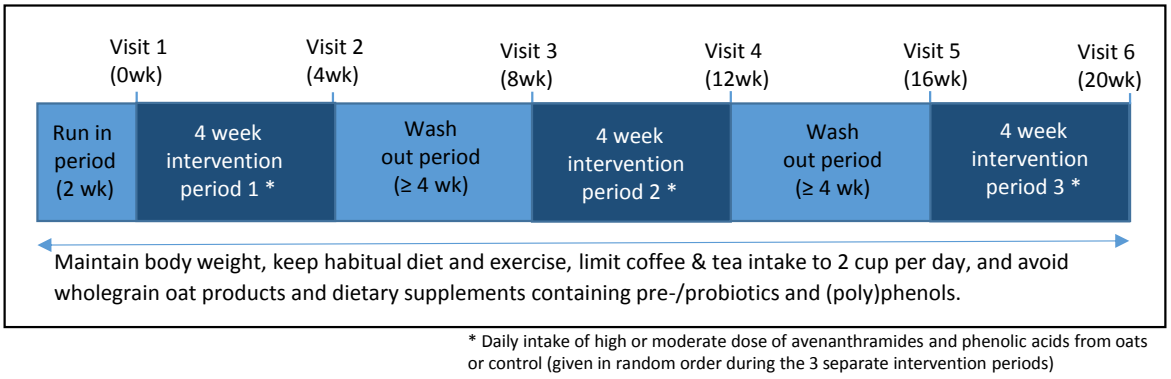


Figure 4.1 Study design

4-week intervention periods and attended 6 study visits at the Nutrition Unit (visits 1, 3, 5 on the first day of each period; visits 2, 4, 6 on the last day of each period).

Food and Content (g)	Control			Moderate Phenolic Oats Intervention			High Phenolic Oats Intervention		
	Breakfast	Afternoon Snack	Daily Total	Breakfast	Afternoon Snack	Daily Total	Breakfast	Afternoon Snack	Daily Total
Food									
Oatmeal	-	-	-	-	-	-	66.8	-	66.8
Oatwell	-	-	-	17.0	-	17.0	-	-	-
Oat Cake	-	-	-	-	-	-	-	60.0	60.0
Cream of Rice	68.8	-	68.8	63.3	-	63.3	-	-	-
Cream Cracker	-	60.0	60.0	-	60.0	60.0	-	-	-
	-	-	-	-	-	-	-	-	-
Nutritional Content									
Energy (kcal)	274.0	226.2	500.2	274.0	226.2	500.2	239.1	261.0	500.1
Cellulose	8.1	0	8.1	0	0	0	0	0	0
Pectin	4.8	0	4.8	0	0	0	0	0	0
Total polyphenols	7.4	6.4	13.8	32.0	6.4	38.9	-	-	68.1
Phenolic acid (mg)	7.4	6.4	13.8	32.0	6.4	38.4	25.5	23.3	48.8
Avenanthramide (mg)	0	0	0	0.5	0	0.5	13.6	5.7	19.3
β-glucan	0	0	0	4.8	0	4.8	2.7	2.2	4.9
Carbohydrate	55.04	45.2	101	52.7	45.2	97.9	37.8	30.8	68.7
Total Fibre	12.9	2.6	15.5	8.8	2.6	11.4	6.8	8.7	15.5
Soluble fibre	4.8	0	4.8	4.8	0	4.8	4.8	0	4.8
Insoluble fibre	8.1	2.6	10.7	4.0	2.6	6.6	10.7	0	10.7
Protein	4.7	6.5	11.2	8.2	6.5	14.7	9.5	7.3	16.9
Total Fat	-	1.6	1.6	0.7	1.6	2.3	4.0	10.1	14.1
Saturated Fat	0	1	1.0	0.1	1	1.1	0.6	1.14	1.8
MUFA	0	0.2	0.2	0.4	0.2	0.6	1.4	6.6	8.0
PUFA	0	0.3	0.3	0.3	0.3	0.6	1.8	1.8	3.6
Sodium	0	1.0	1.0	0	1	1.0	0	0.4	0.4

Table 4.1 Nutritional content and quantities of Intervention Food Materials

Quantities of food stuffs including the intervention materials, phenolic acid*, avenanthramide* and β-glucan, and their nutritional content are shown in grams (g) unless specified as mg or kcal; for the control, moderate and high phenolic oats interventions.
Macronutrients, total fibre and sodium contents quantified by Campden BRI group, UK. Phenolic acid, avenanthramide, β-glucan and energy contents of the food materials are estimates from the literature and food labels. Figures are rounded to one decimal place.
MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids.
* Highlighted blue.

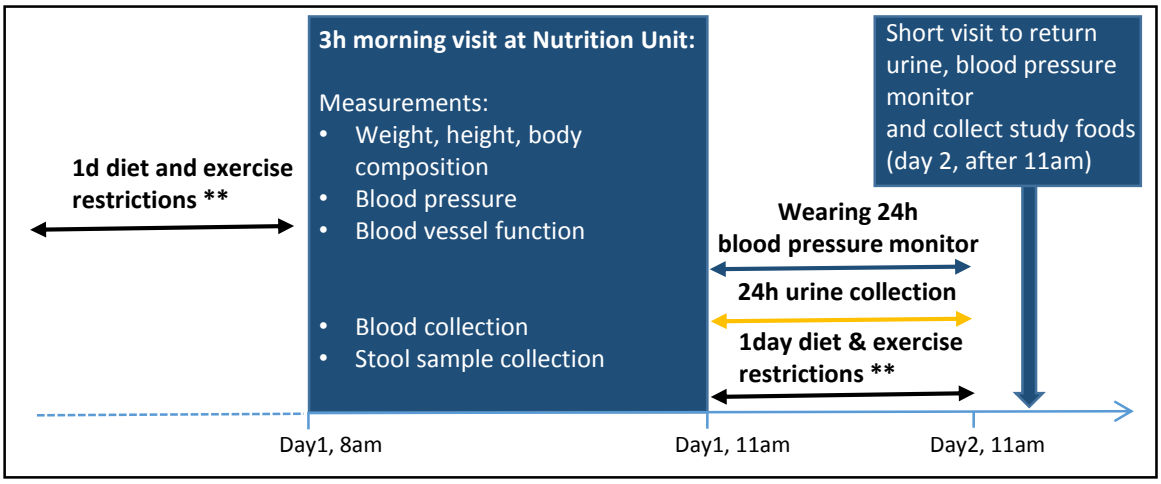
4.2.3 STUDY VISITS

For each study visit volunteers were requested to arrive at the Hugh Sinclair Unit of Human Nutrition in the morning. Volunteers were asked, in preparation for the visit, to fast overnight (i.e. no intake of food or drink for 10h before the visit, except water), maintain complete dietary record sheets and to restrict their diet as detailed in Appendix 9, which included refraining from consuming (poly) phenol-rich foods, moderating consumption to ≤ 21 units of alcohol/week and to refrain in exercise 48h prior to the study visits. On arrival volunteers completed a researcher-administered questionnaire to monitor any potential adverse events and changes in medication, and to collect a full dietary record from dietary record sheets and to verify adherence to the lifestyle restrictions.

Anthropometrical measures were performed (height, weight and % body fat composition (TANITA, UK, Ltd.)), following which the volunteers rested for 20 minutes in a supine position in a temperature-controlled (22 ± 1 C), light-dimmed room and oscillometric blood pressure, FMD, vascular reactivity (LDI) and pulse wave analysis measurements was performed, following standard procedures. Blood was collected and immediately processed, also following standardised procedures. At the end of the visit, volunteers were asked to wear an ambulatory blood pressure monitor for 24h, to collect their urine for 24h and to avoid intake of (poly) phenols, caffeine, alcohol and nitrite/nitrate (Figure 4.2).

On visits 1, 3 and 5, the intervention products for the 4-week intervention period, together with the intake instructions, were provided. A polyphenol-low breakfast (white bread toast, butter, cheese, and water) was provided while the volunteers were in the Hugh Sinclair Unit. During a 2 week run-in period and for the duration of the study, volunteers were asked to completely refrain from eating oats (apart from the study interventions), taking dietary, probiotic or prebiotic supplements, to drink no more than 400 ml/d of tea or polyphenol-low coffee (i.e. a highly roasted polyphenol-degraded commercial instant coffee provided by the researchers) but otherwise to maintain their habitual dietary and activity patterns, and to keep their body weight within 1 kg of their starting weight.

Compliant intake of the intervention materials was monitored using volunteer-maintained intake logs and returned, empty material sachets. Furthermore, urinary avenanthramide excretion was measured as a marker of oat intake.



** Restrictions include: no strenuous exercise and no intake of a range of foods (see list on last page). On the evening before the visit, we will ask you to consume a provided dinner and then fast ≥12h.

Figure 4.2 Volunteer's schedule for each visit

4.2.4 Randomisation and Masking

To minimise bias both researchers and subjects were 'blinded' to the food (intervention) product. Scientists not involved in the study generated random number sequences, and managed volunteer allocation and intervention meal administration. Volunteers were randomly allocated to the intervention sequence (i.e. experimental intervention followed by control intervention or vice versa).

The breakfast products were packaged and labelled by the company in individually weighed sachets that displayed the product ID, ingredient statement, PEP protocol number, repacked date, expiration date, researcher name, telephone number and investigational use statement. Study Product Intake Logs were completed upon the dispensation of the study product to each subject.

4.2.5 Anthropometric Measures

a) Anthropometric measurement

Measurements were taken as described in Chapter 3, Section 3.2.7.

b) 24 h Blood Pressure Measurement

Ambulatory Blood Pressure monitoring (ABPM) was undertaken using a non-invasive ABP monitor (TM-2430, Scan Med, A&D Medical, UK) linked to a computer, and configured using

Doctor Pro TM-2430-13 (version 2.40, A&D Company limited, Japan) software. Blood pressure examinations were undertaken at the same time following vascular measurements and breakfast using ABPM, over a 24 h period following visits to the clinic unit [671]. The monitor was operated according to the guidelines of the British Hypertension Society, to record BP and heart rate at 30 minutes intervals from 07:00 h to 21:59 h and at 60 minute intervals from 22:00 h to 6:59 h, the two initial measurements were discarded [672]. Participants noted activities and sleep hours throughout the recording period in a diary provided for the purpose (Appendix 10).

ABPM is an important technique to measure blood pressure levels over a period of time, which is used to avoid white coat responses in some individuals and to accurately diagnose hypertension [673] and is considered a suitable method of measurement and more accurate than measures used for diagnostic classification [112]. This system's accuracy has been confirmed across the entire range of blood pressure levels and ages, and its efficiency does not fluctuate with gender [674]. ABPM is a non-invasive measurement achieved through an oscillometric process in which external cuff pressure is increased to the point of occluding the brachial artery in the arm. Once the pressure in the cuff is higher than the blood pressure inside the artery, the artery collapses and as the cuff pressure is decreased to below SBP, blood starts to fluctuate through the arterial wall which expands with every pulse. Pressure measured in the cuff at the time the blood first passes through the occluded artery is an estimate of systolic pressure, whereas the pressure when blood first starts to flow continuously is an estimate of diastolic pressure [112]

c) *Flow-mediated dilatation (FMD)*

d) *Laser Doppler Iontophoresis (LDI)*

Measurements were undertaken as described in Chapter 3, Section 3.2.7 for FMD and LDI.

4.2.6 DIET DIARY ANALYSIS

A diet diary is a tool to identify foods, quantify the portion size and determine the frequency of consumption in volunteers' daily eating patterns over a given period. In the three days before each pre-intervention visit (1, 3 and 5), volunteers recorded all the food and drinks they consumed for four consecutive days, one during the weekend and the other three during the week. Records were made on the same days of the week for each diet diary. Data for each diary was analysed using the Dietplan (Version 7) software package, using the McCance, Widdowson and ID2 databases, which contain an extensive list of nutritional information for a

variety of foods available in the UK. The nutrients compared were: saturated; Polyunsaturated and monounsaturated fat; carbohydrate include fibre; protein and sodium intake.

4.2.6.1 BLOOD COLLECTION

Venous blood was collected at every assessment visit by an experienced, qualified researcher, 48 ml was taken per visit. Blood was kept in vacutainers with or without anticoagulants (sodium citrate, lithium heparin, EDTA), as suitable for subsequent analyses. No anticoagulants were used for serum isolation. Plasma EDTA (Ethylenediaminetetraacetic acid) (Geriner BioOne Ltd) was kept in ice and then centrifuged at 4 °C and 2000 RCF for 10 minutes and within 60 minutes of blood collection. Serum vacutainers (rapid serum separator tubes) were kept at room temperature (RT) for 30 to 60 minutes to enable the blood to clot and then centrifuged at 4 °C and 2000 RCF for 10 minutes. Serum and plasma separates were aliquoted into pre-labelled microtubes and immediately stored at 80 °C until further analysis.

4.2.6.2 BIOCHEMICAL MEASURES

a) Blood Lipids

Total cholesterol (TC), Triglycerides (TAG) and HDL cholesterol (HDL-C) were assessed at every visit. Quantification was undertaken with an automatic clinical chemistry analyser (ILAB 600, Instrumentation Laboratory, Warrington, UK), with the help of enzyme-based colorimetric specific kits and serum controls provided by Werfen Limited, UK. Each run included control samples, which contained high and low concentrations of each biochemical parameter, using serum 1 and serum 2 controls. LDL-C (Low-density lipoprotein cholesterol) was determined by means of the Friedewald formula, i.e. ((LDL-C = (TC –HDL-C) – (TAG)/2.2)). Quality control was assured by comparison with samples supplied for this purpose by manufacturer.

b) ACE Activity

c) ACE Concentration

d) Renin Concentration

Measurements for B, C and D were undertaken as described in Chapter 3, Section 3.2.7.

4.2.6.3 URINARY MINERAL EXCRETION

After every visit, volunteers were requested to collect urine for 24 h. Urine volumes were recorded and 4 aliquots kept at -80 °C after centrifugation at 1700 g (3000 rpm) for 15 minutes at 4 °C. Mineral excretions were quantified using flame atomic absorption spectrometry as described below. The metal ions being investigated were measured by comparing their atomic spectroscopic signal with that of a measured solution of the same ion. Mineral element

excretions, including Na and K, were measured similarly. Standard flame atomic absorption spectrophotometry stock solutions of the diverse metal ions, at a concentration of 1000 mg/l (1000ppm), were obtained from (Cole-Parmer Staffordshire, UK) and used to prepare effective solutions by suitable dilutions: 1,.8,.6,.4,.2 and 0 ppm. Glassware was cleansed with Ultrapure de-ionized Milli-Q water prior to use, in order to avoid potential contamination. Measurements were conducted using an atomic absorption spectrometer (NovAA® 350, Analytik Jena AG, Germany), with single and double beam optics and a fully automated 8-lamp turret, for high sample throughput, and equipped with an air-acetylene flame burner.

4.2.6.4 Sample Size and Statistical Analysis

The power calculation was made for the primary clinical outcome measure (FMD of the brachial artery) to determine the minimum number of participants required for the study. The minimal measurable, statistically significant improvement on FMD is an absolute change of between 1.5 to 2 %, considering a baseline vasodilatation of 6-7 % in healthy males. This has taken into account the statistical limitations related to our primary measure of vascular function. The sample size was calculated based on the variance of repeated measurement in the control group and on control data. Consequently, with a standard deviation within participants of 2.4 % (based on previous studies performed in our group), a significance level of $P \leq 0.05$ and a power of 80 %, 27 subjects were needed to determine a significant within-subject difference between interventions of at least 1.3 % of FMD. However, to provide power to use the secondary measures and achieve significant within-subject differences, we aimed for 30 participants to complete the trial. A total of 33 volunteers were, therefore, recruited to allow for a drop-out rate up to 10 %.

To identify differences in the study endpoints (i.e. markers of CVD risk, RAAS enzyme levels and activities and minerals excretions in biological samples) between the assessment time points and interventions, a repeated measures model was used with baseline values as a covariate while the variables of time, intervention and interaction between time and intervention used as the fixed effects. Where the repeated measures model showed significant differences, pairwise comparisons between the interventions and time points were performed using *post hoc* analysis (Tukey adjustment). Only effect was considered when significant level was at a probability of P value ≤ 0.05. All statistical analyses were carried out using the statistical package for social science (SPSS) version 24.

4.3 RESULTS

4.3.1 SUBJECT COHORT AND SELECTION

The procedures for selection and assessment of subjects who were both eligible and suitable to undertake the study, as described in Section 4.2.1, resulted in 87 volunteers of whom 28 completed the entire study. The process which led to this result, and the elimination of 59 suitable subjects following the initial assessment and recruitment procedure, is shown in Figure 4.3.

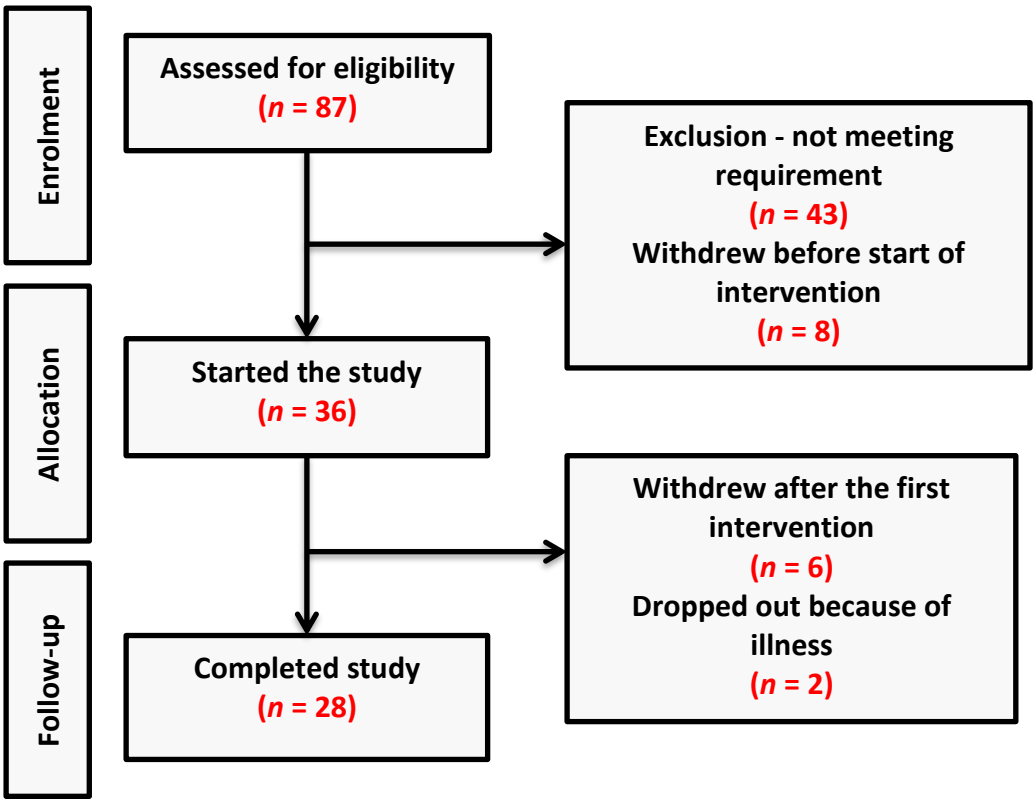


Figure 4.3 Flow diagram for the recruitments and retention of study volunteers

4.3.2 SUBJECT CHARACTERISTICS

Baseline physical characteristics and cardiovascular indicator measurements of the 28 pre-hypertensive or stage 1 hypertensive, mixed gender individuals who completed the study are shown in Table 4.2. Characteristics were measured following overnight fasting. The intervention and control food stuffs were well tolerated by all the volunteers and without any observed side effects.

Characteristic	Mean ± SEM	Range
Age (y)	49.6 ± 2.3	26 - 68
BMI (kg/m^2)	26.7 ± 0.7	20.9 - 39.8
Systolic blood pressure (mm Hg)	129.7 ± 1.9	112-153
Diastolic blood pressure (mm Hg)	80.1 ± 1.2	65 - 96
Heart rate (bpm)	71.1 ± 2.3	55.0 - 107
Plasma glucose (mmol/L)	5.6 ± 0.1	3.9 - 7.7
Plasma total cholesterol (mmol/L)	5.3 ± 0.1	3.4 – 7.1
Haemoglobin (g/L)	145.5±2.1	126-177

Table 4.2 Measured and physical characteristics of overnight fasted study participants at screening.
BMI – Body Mass Index; n = 28

4.3.3 FLOW-MEDIATED DILATATION (FMD)

Effects on FMD due to the phenolic oats interventions is a primary expected outcome of this study. In the control, moderate- and high phenolic oats interventions, % FMD responses were improved from baseline measurements by increases of 0.43, 0.50 and 1.09 % respectively (Figure 4.4, calculated from Table 4.3) after 28-days of consumption; however, there was no significant difference between the three groups (P = 0.520). There was, however, a highly significant difference (P = 0.007) in % FMD, in all intervention groups combined, between the baseline and post-intervention times. The interaction between intervention × time was not significant (P = 0.812). Hence, it appears that any intervention, including the control which included low phenolic oats, but was energy and fibre balanced, leads to an improvement in vascular reactivity measured by % FMD over time, but that the level of the phenolic oat intervention, in these trial conditions, was not significant.

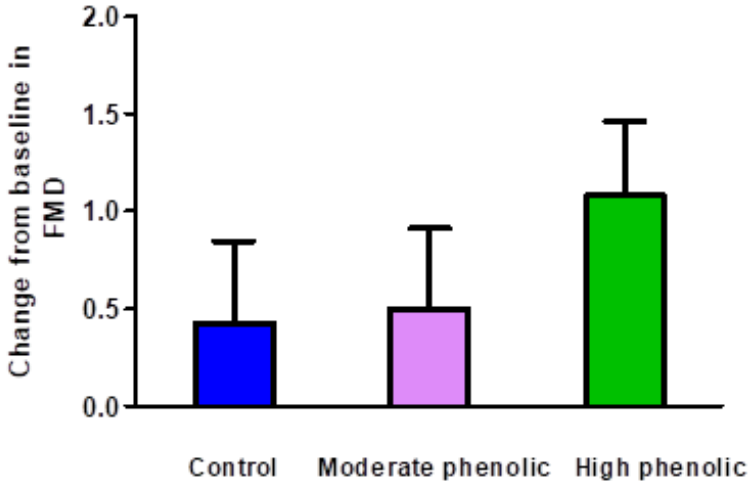


Figure 4.4 Changes from baseline in FMD at the end of the 4-week intervention period

High, Moderate and Control (low avenanthramide and phenolic acid) intervention groups. Thick bars – means; thin bars – SEMs; (n = 28).

Vascular Reactivity Measure (pu/h^{-1})	Control		Moderate Phenolic Oats Intervention		High Phenolic Oats Intervention		Probabilities (P)		
	Baseline	Post	Baseline	Post	Baseline	Post	Interven-tion	Time	I × T
FMD (%)	5.698 ± 0.418	6.125 ± 0.482	5.647 ± 0.416	6.148 ± 0.412	5.763 ± 0.383	6.851 ± 0.471	0.520	0.007	0.812
LDI-Ach AUC	1188.65 ± 233.23	1156.49 ± 233.4	1312.84 ± 171.5	1290.9 ± 236	1569.64 ± 227.13	1716.3 ± 292.9	0.552	0.337	0.868
LDI-SNP AUC	1171.8 ± 200.77	1233.3 ± 150.7	1127.8 ± 127.3	1412.8 ± 256.8	1459.4 ± 241.6	2011.5 ± 386	0.221	0.037	0.363
LDI-Ach iAUC	320.7 ± 163.4	298.22 ± 137.6	378.12 ± 135.6	344.5 ± 207.2	611.3 ± 220.4	655.0 ± 207.7	0.467	0.249	0.798
LDI-SNP iAUC	407.22 ± 172.39	353.1 ± 114.4	255.6 ± 65.11	406.15 ± 161.2	492.0 ± 206.3	872.1 ± 286.6	0.271	0.038	0.682

Table 4.3 Vascular measurements pre- and post-intervention for Phenolic Oats interventions.

Vascular reactivity measured pre-intervention (Baseline) and week 4 post-intervention (Post), for the control, moderate and high phenolic oats interventions. Phenolic oats comprise avenanthramide and phenolic acid. A repeated measures model used for dose and time dependent differences between baseline and post-intervention measurements, for each intervention group. Significant results ($P < 0.05$) are highlighted pink. Pairwise comparisons between interventions and time points were performed using *post hoc* analysis (Tukey). All values are means ± SEMs, n = 28. pu/h^{-1} – perfusion units per hour.
FMD – Flow-Mediated Dilation; LDI - Laser Doppler Iontophoresis; Ach – Acetylcholine; SNP - Sodium Nitroprusside.

4.3.4 LASER DOPPLER IONTOPHORESIS (LDI)

Microvascular reactivity measured using Laser Doppler Iontophoresis (LDI) is a secondary expected outcome of this study, measured by looking at changes in endothelium-dependent (using acetylcholine (LDI-Ach)) and endothelium-independent (using Sodium Nitroprusside (LDI-SNP)) microvascular blood flow, in volunteers fasted overnight. However, regular intake of phenolic oats for a 28-day period led to no significant differences in any mean LDI measurements (Table 4.3 above), in comparison to the control group intake, nor was there any significant differences when intervention was combined with time from baseline to post-intervention (I × T, Table 4.3).

Considering changes between the baseline and post-intervention results for the three interventions combined (shown as Time in Table 4.3), no significant changes were detected by LDI in skin blood flow in response to endothelium-dependent microvascular reactivity using LDI-Ach, expressed as either area under curve (AUC) or incremental area under curve (iAUC) measures (P = 0.552 and P = 0.467 respectively) (Figure 4.5, Table 4.3). However, there were, small but measurable increases in both measures, for the high-dose intervention of 146.66 and 43.7, (all ±SEM, pu/h^{-1}, calculated from Table 4.3); this result is indicative of a trend only, given the lack of significance. Similar analysis for endothelium-independent microvascular reactivity using LDI-SNP, showed a significant improvement over time, expressed as both AUC and iAUC (P = 0.037 and P = 0.038, respectively), between the baseline and post-intervention results, for all three interventions combined (Figure 4.6, Table 4.3), this result reflects the significant change in the primary outcome, % FMD, over time and therefore is likely to represent a medically relevant finding. Increases were found, albeit clearly non-significant and, therefore, also cautionary, in both measures (P = 0.221 and P = 0.271 respectively), for both the moderate- and high-dose interventions: moderate AUC/iAUC 285.0 and 552.1; high AUC/iAUC 150.55 and 380.1 pu/h^{-1} (all ±SEM, calculated from Table 4.3).

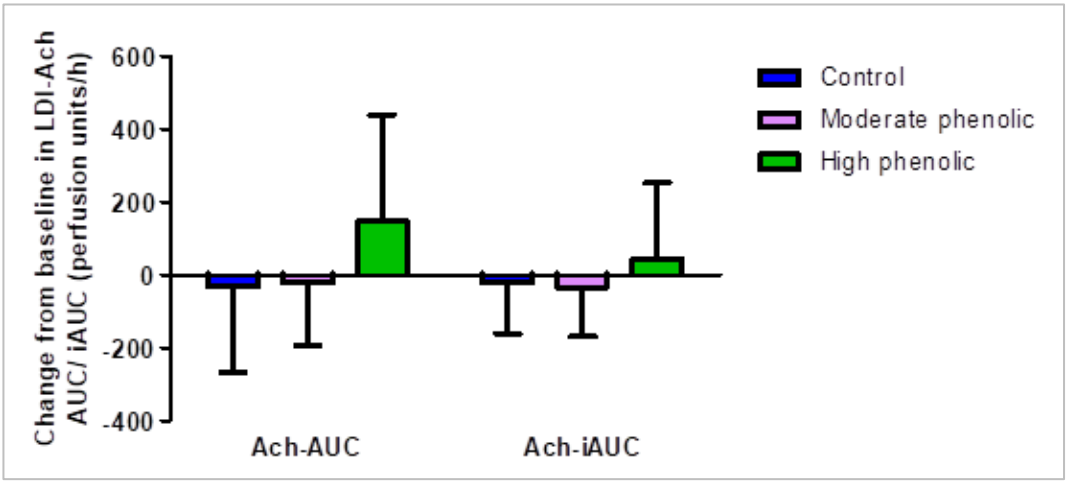


Figure 4.5 Changes from baseline in LDI-Ach AUC and iAUC at the end of the 4-week intervention period

Intervention groups: control (blue), moderate phenolic oats (pink) and high phenolic oats (green) interventions. Thick bars – means; thin bars – SEMs; (n = 28). Results were not significant (CI = 95%)

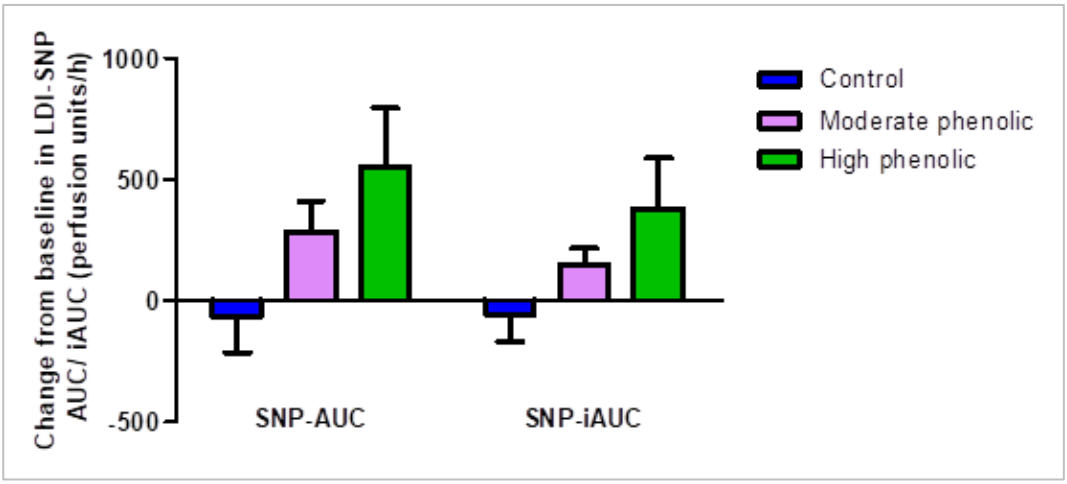


Figure 4.6 Changes from baseline in LDI-SNP AUC and iAUC at the end of the 4-week intervention period

Intervention groups: control (blue), moderate phenolic oats (pink) and high phenolic oats (green) interventions. Thick bars – means; thin bars – SEMs; (n = 28). Results were not significant (CI = 95%)

4.3.5 TWENTY-FOUR HOUR AMBULATORY BLOOD PRESSURE (ABPM)

The evaluation of 24 hour ABPM, full 24 hour SBP and full 24 hour DBP, analysed by a repeated measures model, showed no significant differences between the control, moderate and high phenolic oat interventions. Differences in the time between baseline and post-intervention measures and the interaction between intervention × time (Table 4.4, Figure 4.7) also showed no significant differences. However, analysis of just daytime measurements of both SBP and DBP showed a similar, significant reduction occurred between baseline and post-intervention measurements ($P = 0.047$ and $P = 0.048$ respectively), which is indicative only as this is a secondary outcome of the trial. There was no difference arising from the intervention dosage.

Consumption of high doses of avenanthramide and phenolic acid oats led to a possible fall in full 24-hour SBP, by 1.16 mm Hg on average, whereas a moderate dose intervention and the control failed to lower SBP, all results were not significant so must be treated with caution. Night-time SBP reduced by 5.1 mm Hg from 115.68 mm Hg at baseline to 110.58 mm Hg after high phenolic oat intervention, compared to a reduction of only 0.52 mm Hg in the moderate intervention and no reduction in the control group. However, night-time SBP changes were also not statistically significant between interventions, time (baseline to post-intervention) or the interaction between them ($P = 0.832$, $P = 0.250$ and $P = 0.488$ respectively). A non-significant and, therefore, only suggested reduction by 2.26 mm Hg was also found in night-time DBP for the high avenanthramide and phenolic acid intervention group, between the baseline and post-intervention measurements, whereas the moderate and control groups showed smaller reductions, by only 1.06 and 0.98 mm Hg respectively (Table 4.4, Figure 4.7).

Blood Pressure Measure (mm Hg)	Control		Moderate Phenolic Oats Intervention		High Phenolic Oats Intervention		Probabilities (P)		
	Baseline	Post	Baseline	Post	Baseline	Post	Intervention	Time	I × T
24 h SBP	125.77 ± 1.75	129.27 ± 2.36	126.19 ± 2.27	128.77 ± 2.75	127.39 ± 1.67	126.23 ± 1.53	0.848	0.228	0.116
Day SBP	129.68 ± 1.89	134.85 ± 2.37	129.54 ± 2.23	134.00 ± 3.04	131.73 ± 1.82	131.58 ± 1.95	0.807	0.047	0.234
Night SBP	113.20 ± 2.21	114.48 ± 2.34	114.17 ± 2.66	113.65 ± 2.41	115.68 ± 1.68	110.58 ± 2.05	0.832	0.250	0.488
24 h DBP	76.77 ± 1.24	77.88 ± 1.32	77.50 ± 1.29	79.12 ± 1.47	77.65 ± 0.94	78.38 ± 0.98	1.000	0.151	0.816
Day DBP	79.96 ± 1.10	81.46 ± 1.30	80.42 ± 1.38	83.19 ± 1.59	80.95 ± 0.95	81.92 ± 1.19	0.993	0.048	0.676
Night DBP	68.44 ± 1.65	67.46 ± 1.25	68.83 ± 1.59	67.77 ± 1.51	68.68 ± 1.12	66.42 ± 1.51	0.939	0.306	0.977

Table 4.4 Ambulatory Blood Pressure Measurements pre- and post-intervention for Phenolic Oats interventions.

Twenty-four-hour ambulatory blood pressure measured pre-intervention (Baseline) and week 4 post-intervention (Post), for the control, moderate and high phenolic oats interventions. Phenolic oats comprise avenanthramide and phenolic acid. A repeated measures model used for dose and time dependent differences between baseline and post-intervention measurements, for each intervention group. Significant results ($P < 0.05$) are highlighted pink. Pairwise comparisons between interventions and time points were performed using *post hoc* analysis (Tukey). All values are means ± SEMs, n = 28.
SBP – Systolic Blood Pressure; DBP – Diastolic Blood Pressure.

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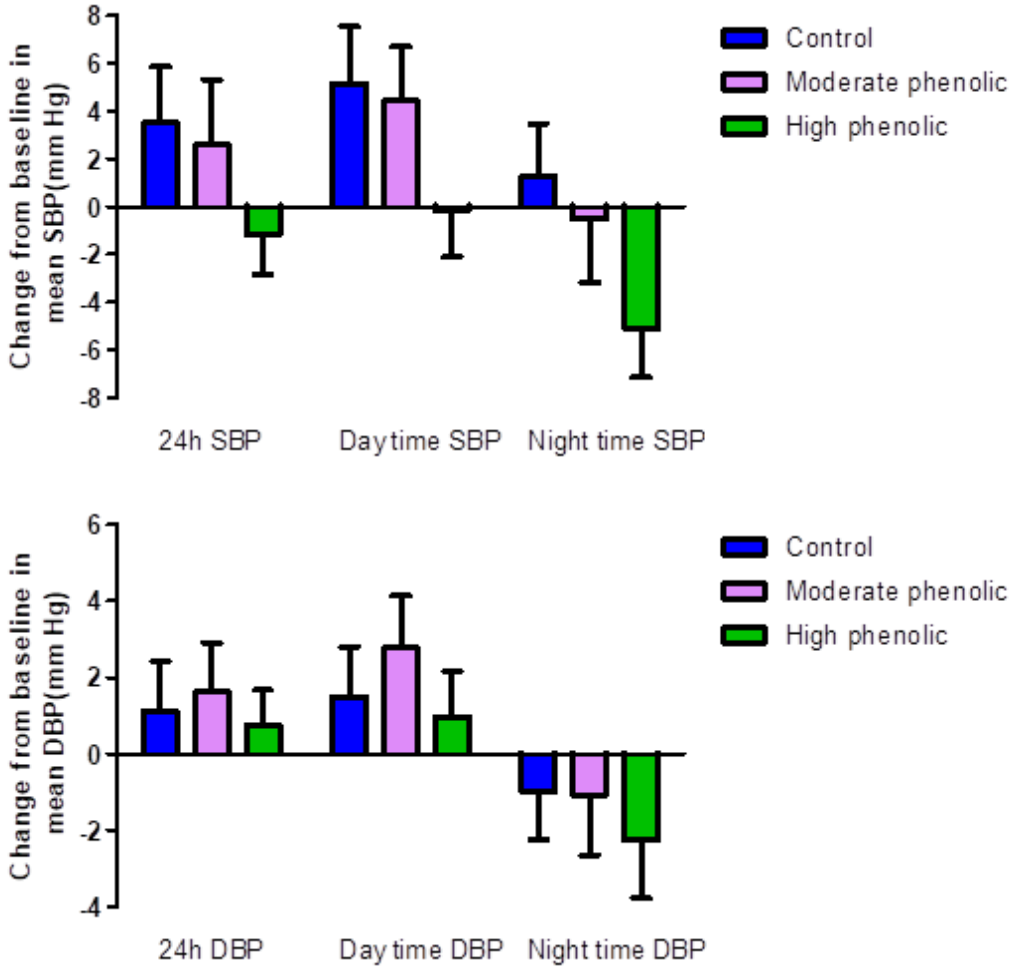

Figure 4.7. Changes in 24 hour, day-time and night-time systolic (A) and diastolic (B) ambulatory blood pressure (BP)

Measurements taken at the beginning and end of the 4-week intervention period for the control, moderate phenolic oats and high phenolic oats intervention groups. Phenolic oats comprise avenanthramide and phenolic acid. Thick bars – means; thin bars – SEMs; (n = 28).

4.3.6 RAAS Enzyme Levels and Activity

In a repeated measures GLM, no significant differences in ACE levels, ACE activity or plasma renin levels were observed between the control, moderate or high phenolic oats interventions. Differences between baseline and post 4-week intervention, and for the interaction between intervention × time were also non-significant (Table 4.5). Results suggest, albeit with weak statistical support, that renin and ACE levels dropped in the control, between baseline and post-intervention measurements, however, ACE activity rose slightly. The reverse occurred in the moderate intervention group, with only ACE activity dropping, by 0.19 ng/mL). The high phenolic oat intervention group followed the pattern of the control, with ACE levels and renin levels reduced by 0.37 and 28.03 ng/mL respectively, ACE activity also dropped in high phenolic oats by 2.4 U.

4.3.7 Lipid Profile Measures

A repeated measures GLM showed no significant differences in any of the measured lipid indicators between the control, moderate or high phenolic oats interventions (Table 4.5). However, significant differences in the baseline and post-intervention (time) measurements were found in total (P = 0.033) and HDL (P = 0.01) cholesterol. Small, non-significant and therefore only indicative reductions were observed in lipid profiles. TC fell by 0.2 and 0.16 mmol/L in the moderate and high phenolic oat interventions between the baseline and post-intervention measurements, compared to a slight increase in the control group. LDL decreased by 0.07 and 0.26 mmol/L respectively, compared to 0.05 in the control. HDL also decreased by 0.14 and 0.04 mmol/L respectively, compared to 0.02 in the control. Triglycerides increased slightly, by 0.17 and 0.05 mmol/L respectively, compared to 0.06 in the control All figures calculated from Table 4.5. However, lipid measures are a secondary outcome and the trial was not tested for power beyond the primary outcome.

4.3.8 Mineral - Sodium (Na) and Potassium (K) Excretion

The interaction (intervention × time, Table 4.5) between phenolic oat intervention and time between baseline and post-intervention measurements, was found to be significant (P = 0.04) in the measurement of Na (urinary salt, NaCl) excretion, but not K, but since this is a secondary trial outcome and intervention was non-significant for % FMD, therefore, as this is a possibly under-powered trial, the results may not be reliable. There were no observed significant differences between the control and the moderate or high phenolic oats groups. Nor were there significant differences between the baseline and post-intervention (time) measurements for these minerals. While the levels of Na and K excretion putatively fell in the high phenolic oats treatment group, the fall was smaller than the control group for Na (8.51 compared to 8.62 mmol/L), but larger in K (15.95 compared to 2.48 mmol/L). Both Na and K excretions also rose, putatively in the moderate phenolic oats intervention

group, 13.29 and 4.32 mmol/L respectively, however, the caveats with respect to reliance on secondary outcomes pertain.

Lipids, Minerals and RAAS Enzymes	Control		Moderate Phenolic Oats Intervention		High Phenolic Oats Intervention		Probabilities (P)		
	Baseline	Post	Baseline	Post	Baseline	Post	Intervention	Time	I × T
Total cholesterol (mmol/L)	5.20 ± 0.18	5.28 ± 0.17	5.37 ± 0.17	5.17 ± 0.19	5.24 ± 0.15	5.08 ± 0.17	0.887	0.033	0.442
LDL cholesterol (mmol/L)	3.15 ± 0.15	3.10 ± 0.19	2.97 ± 0.26	2.90 ± 0.22	3.18 ± 0.12	2.92 ± 0.18	0.681	0.319	0.789
HDL cholesterol (mmol/L)	1.51 ± 0.07	1.49 ± 0.07	1.63 ± 0.08	1.49 ± 0.07	1.54 ± 0.07	1.50 ± 0.07	0.786	0.010	0.650
Triglycerides (mmol/L)	1.19 ± 0.08	1.25 ± 0.12	1.03 ± 0.07	1.20 ± 0.10	1.16 ± 0.09	1.21 ± 0.10	0.773	0.129	0.817
Na excretions (mmol/L)	97.60 ± 9.36	88.98 ± 7.43	93.66 ± 6.90	106.95 ± 7.54	97.97 ± 6.53	89.46 ± 5.97	0.723	0.746	0.040
K excretions (mmol/L)	41.26 ± 5.61	38.78 ± 3.24	40.83 ± 3.45	45.15 ± 4.11	51.16 ± 14.47	35.21 ± 2.23	0.906	0.425	0.353
Renin level (ng/mL)	752.64 ± 81.40	710.27 ± 80.16	910.89 ± 140.29	917.96 ± 136.04	818.46 ± 132.88	790.43 ± 112.23	0.548	0.381	0.694
ACE level (ng/mL)	157.05 ± 8.98	152.20 ± 8.23	148.89 ± 8.23	151.23 ± 9.58	149.65 ± 9.14	149.28 ± 9.51	0.912	0.657	0.621
ACE activity (U)	37.01 ± 3.38	38.86 ± 3.60	38.64 ± 3.42	38.45 ± 2.95	39.31 ± 2.86	36.91 ± 3.26	0.990	0.871	0.514

Table 4.5 Fasting lipid profile, mineral excretions and RAAS enzymes Measurements pre- and post-intervention for Phenolic Oats interventions.

Fasting lipid profile, mineral excretion and RAAS enzymes measured pre-intervention (Baseline) and week 4 post-intervention (Post), for the control, moderate and high phenolic oats interventions. Phenolic oats comprise avenanthramide and phenolic acid. A repeated measures model used for dose and time dependent differences between baseline and post-intervention measurements, for each intervention group. Significant results ($P < 0.05$) are highlighted pink. Pairwise comparisons between interventions and time points were performed using *post hoc* analysis (Tukey). All values are means ± SEMs, n = 28.

LDL – low-density lipoprotein; HDL - high density lipoprotein; Na – Sodium; K – Potassium; ACE – Angiotensin Converting Enzyme.

4.3.9 NUTRIENT INTAKE SUMMARY

Although there were differences in the dietary intake of carbohydrate, fat and MUFA, there were no statistically significant differences observed between the groups for these nutrients ($P > 0.05$) (Table 4.6).

Nutrient (g)	Control Intervention	Moderate Phenolic Oats Intervention	High Phenolic Oats Intervention	Range
Energy (kcal)	2338 ± 120	2328 ± 133	2456 ± 165	2195 - 2621
Carbohydrate	311 ± 18	305 ± 19	287 ± 16	271 - 329
Fibre	33 ± 2	29 ± 2	33 ± 2	27 - 35
Protein	95 ± 5	99 ± 6	107 ± 7	90 - 114
Total Fat	75 ± 4	76 ± 6	92 ± 9	71 - 101
Saturated Fat	28 ± 2	28 ± 3	31 ± 3	25 - 34
MUFA	24 ± 2	24 ± 2	31 ± 3	22 - 33
PUFA	10 ± 1	11 ± 1	14 ± 1	9 - 15
Sodium	3.1 ± 0.2	2.9 ± 0.3	3.4 ± 0.3	2.6 - 3.7

Table 4.6 Daily intake - dietary comparison between intervention groups

Daily consumption of the principal dietary nutrients is shown for the control, moderate and high phenolic oats interventions. Phenolic oats comprise avenanthramide and phenolic acid.
MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids. All values are means ± SEMs, n = 28.

4.4 DISCUSSION

Of the numerous published epidemiological and human intervention studies concerning the cardiovascular effects of oats, the overwhelming focus has been on the potential benefits of increased whole grain or whole grain fibre consumption, in particular β-glucan. In this study we have highlighted the vasoactive potential of the intake of medium and high phenolic acid containing oats in adults, over a chronic, 4-week intake period, relative to an iso-caloric control. Our results indicate that the consumption of oats at our highest dosage of phenolic acids (66.8 g of oatmeal and 60 g of oat cake; 48.8 mg phenolic acid and 19.3 mg avenanthramides) tended to improve 24 h systolic BP, day and night time BP and endothelial microvascular blood flow, relative to the control, although these effects did not achieve statistical significance and are of limited reliability as secondary outcomes, given the power calculation for this trial. Despite the lack of statistical significance, the magnitude of the mean reduction in BP is tentatively proposed as medically relevant (24-hour SBP reduced by 1.16 mm Hg on average, night-time SBP reduced by 5.1 mm Hg). In addition, the, also non-significant, enhancement in microvascular blood flow observed following the intake of the high phenolic oat intervention might manifest as maintenance of daytime BP, relative to the control and the lower phenolic oat intake groups, since daytime BP was observed to increase in these groups. FMD of the brachial artery and microvascular reactivity were not consistently improved by whole

grain oat intake, which is consistent with a review study which reported that, of 25 whole grain oats studies, only three measured significant reductions in blood pressure [40]. Also, notably, a dose-dependent study in people with slightly elevated blood pressure, suggested that the intake of high flavonoid containing fruit and vegetables did not significantly influence endothelium-dependent or -independent blood flow [342]. Our data indicate, in general, that although there may be a tendency of high phenolic containing oat intake to improve a range of vascular markers, our population size is too small and our dosage may also have been too low to achieve statistical significance.

The relatively recent meta-review of oat-based chronic studies introduced above reported no significant changes in a wide range of CVD risk markers [40], in a large majority of the studies reviewed. We observed that % FMD, our primary outcome, increased significantly over the trial period, but while there were no significant differences between the intervention groups, baseline % FMD increased by 0.43, 0.50 and 1.09 % in response to control, moderate and high phenolic oats interventions, respectively. Oat and wheat based whole grain studies that have found similar low-level responses in FMD include at least one which also specifically targeted hypercholesterolemic patients [667]. Another, larger study of 315 overweight individuals given whole grain oats for four months at two dosages , also found, comparably to our results, just small improvements in endothelial function, between the start and end of the study and no significant differences between interventions [668]. However, significant increases in % FMD by 1.80 ± 0.50% have been reported following the consumption of a wild green oat extract (1500 mg/day), a grain derived extract containing much higher levels of phenolic acids and other potential bioactive substances such as saponins, vitexins and isovitexin [53]. FMD has also been measured in other studies of high-phenolic foods, for example a study using orange juice extract at 200 mg /day, one using both monomeric and oligomeric flavanols from grape seeds and one examining the effect of 100 mg /day of pure epicatechin and 160 mg /dayquercetin-3-glucoside [633, 675, 676]. These three studies all found small but non-significant improvements.

We measured higher endothelium-dependent reactivity following intake of the high and medium phenolic acid oats relative to the control intake, although these changes were secondary outcomes and not significant and, therefore, of indicative only. A whole grain intervention study found similar responses, with differences between dependent and independent vasodilation, not significant [669]. It is tentatively suggested that, despite individual measures of vascular markers being relatively variable in our trial population making statistical significance hard to achieve, the intake of oats with highest phenolic acid content tends to increase endothelium-independent microvascular blood flow and lower blood pressure most.

Our results showed a putative reduction in night-time SBP in the high phenolic oats intervention group of around 5 mm Hg, a decrease regarded as possibly medically relevant from a chronic intervention trial design, however by both the lack of statistical significance and the limitations of a secondary outcome in a trial design that may be under-powered. Notably, the high phenolic acid oats also attenuated a daytime increase in blood pressure observed in both the control and medium-phenolic acid groups. A maintenance of daily daytime blood pressure may signify lower vascular stress, in addition to the potential benefits of a reduction in night time BP [677-679]. Despite these potentially beneficial effects, we further emphasize that in the large part these data failed to achieve statistical significance and as such we are cautious with our conclusions relating to the vascular benefits of this dietary level of oat intake. Whole grain has been shown to induce significant reductions in both SBP and DBP, in slightly hypercholesterolemic volunteers [657] and hypercholesterolemic men [658], during intervention levels and periods similar to our trial. Whole grain oats, in a similar mildly hypertensive study groups to ours [601] found a significant 6 mm Hg reduction in SBP after a 6-week intervention, but, notably, the error in these results was ± 7 mm Hg. Significant reductions, after whole grain oat interventions, of 7.5 mm Hg SBP and 5.5 DBP have also been found [598, 659]. We note that previous clinical trials that have reported statistically significant benefits in vascular health markers, including BP, have predominantly used interventions containing much higher levels of phenolics than our 126.8 g whole grain oats, which contained only 68.1 mg combined phenolic acids and avenanthramides and was designed to represent a realistic dietary intake, achievable with commercially obtainable oats.

One study which presented results comparable to ours also found that ambulatory 24-h BP following oat intake, despite having a longer chronic intervention of 12 weeks, was not significantly changed; this study also pertained to hypertensive men [666]. However, most BP studies investigating whole grain can be regarded as being prone to inaccurate BP reporting, particularly where reliance has been on point-in-time measurements which may miss diurnal changes, and which are prone to "white coat" effect [40]. Several studies have instead, like ours, relied on ABPM, the now more generally accepted approach to measuring BP [40, 671, 680] as it avoids white coat hypertension and incorporates natural diurnal variation.

Another reason for the lack of significance in our results, and smaller effects compared to other studies, might be that phenolic acids, such as ferulic acid and its metabolites, may not be as biologically active as other polyphenols, such as flavonoids. The most convincing evidence so far for improvements in endothelial function from polyphenol consumption has been amassed from studies of foods rich in flavanols, for example cocoa [44] and tea [681-683]. A meta-analysis of 133 chronic cocoa/chocolate trials, reported improvements in FMD by 1.45 %, on average, at flavanol intake

levels of 100-200 mg/day [48]. Also, trials using chronic cocoa (containing epicatechin and catechin levels of 88 mg flavanols) interventions have reported significant improvements in % FMD responses, reductions in 24 h daytime and night-time blood pressure in hypertensive subjects [286]. Phenolic-rich olive oil intake improved endothelial function measured after four months [684]. Endothelium-dependent microvascular relaxation was also shown after 12 weeks in men who received high doses of flavonoids (15 mg/100g) [342]. Consumption of (97.8 mg phenolics) of hibiscus tea for 6 weeks lowered SBP and left DBP unaffected [685], the likely bioactives were gallic acid, delphinidin-3-sambubioside and cyanidin-3-sambubioside, again this was a much higher dose and slightly longer study than ours.

However, evidence of improved vascular function from phenolics in berries such as cranberry and blueberry is also both ambiguous and reliant on abnormally high phenolic dosages. No cumulative effect was found by [334] after chronic cranberry juice consumption by stable coronary artery disease (CAD) subjects at 835 mg/d total polyphenols (TPs) and 94 mg/d anthocyanins, either on FMD or on vascular function in general; while the same authors carried out an open-label pilot study which reported the opposite, with effects on FMD both substantive and favourable. Polyphenol-rich chokeberry juice consumed for a month was also shown to significantly reduce mean 24 h, day systolic and diastolic BP [686] while an acute study of overweight men showed a correlation between improved FMD and consumption of 694 mg/d TPs, including 493 mg anthocyanins from açaí berries [329]. Two blueberry drink studies [49], one investigating time-related effects varied the TP dosage 766 – 1791 mg and the other dose-dependent effects, TP dosage 319 - 1791 mg, found several CVD markers were unchanged with either time or dosage. However, they also showed that while FMD rose significantly at two time points after consumption, 1–2 and 6 h, in the intervention group given 766 mg TC, it was not significantly different in the higher 1278 and 1791 mg groups. In the dosage dependent study, FMD increased linearly up to the 766 mg intake, after which it plateaued and started to decrease slightly at higher doses. This seems to illustrate two points, firstly that while very high dosages may be needed to elicit significant effects on vascular function, there may be an upper threshold above which benefits cease to accrue, and the other that findings in this field are not consistent and, therefore, there are many outstanding questions to be resolved and our non-significant and low-effect results are not unexpected..

The RAAS is an important system controlling BP homeostasis, and cardiovascular and renal function humans and its critical role in probably all CVD has been increasingly recognised in recent years [660]. We found slight, non-significant, reductions of renin and ACE levels of 28.03 and 0.37 ng/mL respectively following high phenolic oat intake which should be treated with caution given the limitations of our trial detailed above. Our data are in putative agreement with previous trials with

blueberry which reported no significant changes in ACE activities over a 3 week period [299], and a trial with grape seed extract for two months which found no significant decreases in plasma renin activity [687]. Significant effects on the mean arterial pressure, SBP and DBP, have also been shown in response to pomegranate intake over a four week period, but serum ACE levels showed no correlation to BP reduction [688, 689]. Similarly, phenolic-rich olive oil has been shown to improve BP, in women, via a mechanism involving RAAS. The mechanism of action may have been through a decrease in serum Ox-LDL and plasma CRP which was measured in subjects after the intervention and hence suppression of the RAAS was likely, with hydroxytyrosol and its derivatives the most likely protagonists [294]. While clinical trials show mixed results with respect to RAAS effects, many *in vitro* studies show strong inhibitory effects of Oat polyphenols on RAAS, particularly ACE inhibition and/or renin expression [374, 661]. Wheat also inhibits ACE activity *in vitro* [662], and has been shown to strongly inhibit ACE levels in blood plasma [663]. Largescale studies have shown that increased urinary sodium and potassium excretion, as proxies for dietary intake, is associated with CVD and other health risks [664, 665]. However, we did not find any significant changes in sodium or potassium excretions between interventions. A 12 week study of the effects of an oat diet on 24h BP in hypertensive men found that 24 hour excretions remained unchanged after intervention [666] and a 5 week whole-grain diet study reduced SBP and DBP but found no differences in urinary excretions of minerals related to BP [657]. However, study of urinary salt excretions, has shown positive effects from a barley diet compared to different whole grain intake in hyperlipidaemic subjects [658].

We found no significant changes in blood lipid profile in any of the measures we used. Our results are consistent with some other studies which have concluded that whole grain diet led to no significant difference in plasma lipid profiles [668, 670], however, our power calculations were calculated based on FMD, the primary study outcome, rather than lipid markers and so our results may be due to lack of statistical power.

In conclusion, we highlight the vasoactive potential of moderate and high phenolic acid and avenanthramide-rich oat consumption in adults over after a 4-week intake period, relative to that of an iso-caloric control low in fibre and phenolics. Our data suggest that at normal dietary intake levels, oats may have a subtle, beneficial influence on the human vascular system, as we find some small improvements in CVD risk markers which were dependent on dosage of phenolic oats, Our observations regarding the small but discernible influence of phenolic oats intake on BP and on the RAAS are to an extent supported by findings reported in the literature, both in scale and in the markers effected. Our results are, however, subject to several caveats. The relatively short duration of the trial, along with the sample size, which was too small to have sufficient power to draw conclusions a cross all the markers tested, are likely the principal limitations. Thies *et al*. (2014),

considered the issue of power in statistical studies, of the 25 relevant studies reviewed, they report none had sufficient power "to rigorously evaluate the effect of oats or oat bran on this outcome" [40] and only five had samples of sufficient size (> 60 per group, assuming 10-20 % baseline variation in subjects, to give 90% experimental power to measure effects of 5-7 %). However, the inconclusiveness of our results is not uncommon amongst comparable studies. Another systematic review, of 21 randomised controlled trials, where the impacts of oat intake were being studied, found only 13 showed significant reduction in TC and only 14 significant reductions in LDL [656]. Lack of compliance with the intervention diet cannot be discounted as another potential factor influencing our lack of statistically relevant findings. We enforced strict controls over dietary intake of Oat but, as with the majority of dietary trials, relied on self-reporting of diet by individuals. Our dietary analysis of the study population indicated no significant differences in the dietary intake of carbohydrate and fat [690], fibre [691] and MUFA [31, 692], dietary components that could influence specific outcomes such as cholesterol and lipid profiles. We may require further study with considerably more participants, and for a longer intervention period, for the effects to become better resolved and statistically significant.

Chapter: 5 General Discussion & Future Perspectives

5.1 STUDY OUTLINE

While there is a substantial body of evidence regarding the health benefits of dietary whole grains and of plant polyphenols, it is nevertheless both incomplete, and contradictory. Oats in particular, while frequently studied in a whole grain dietary context, have rarely been considered for the purveyors of a unique combination of polyphenols which in fact they are. Avenanthramides and phenolic acid, are the principal oat polyphenols, but there is very little published data specific to their efficacy. In the body of published data on polyphenol bioactivity across a range of common foodstuffs, the conclusions regarding the size and nature of their impacts on a wide range of cardiovascular risk markers are widely inconsistent, which may derive from methodological or biomedical causes, but whatever the cause, the picture remains very unclear. BP and a range of endothelial responses have been frequently assayed and are useful markers because they are both an easy demonstration of cardiovascular health and allow cross-study comparisons, since they are generally applicable and easy to measure. However, the impacts of polyphenols and in particular oat polyphenols on the RAAS, despite its well known prominence in proper cardiovascular function, are very much more poorly understood. We clearly do not know how effective oat polyphenols are at preventing CVD, at what dosages they may be effective and what their modes of operation are. This study executed a series of related *in vitro* and human intervention trials designed to provide new evidence to provide more certainty in relation to all of these gaps in our scientific knowledge.

5.2 IN VITRO ASSAYS – THE ROLE OF THE RAAS

Modulation of the RAAS, a critical regulator of BP and vascular function, is one of several proposed mechanisms for polyphenolic action, which may be affected by either inhibited renin expression or ACE activity or both [254, 297, 358, 362, 366, 369]. The RAAS plays a crucial role in controlling BP via production of a vasoconstrictor, Ang II, which has a role in regulating blood volume and vasodilation independently of NO [501, 502]. Renin is the rate limiting step in the RAAS, hence it is believed to be a good target for treatment of hypertension and cardiovascular disease [552]. Likewise, suppression of renin and ACE are two of the strategies used for lowering BP, maintaining electrolyte homeostasis, and for haemodynamics and blood volume status regulation [253, 504]. The aim of this study was to better comprehend the mechanism of action through which polyphenols achieve physiological improvements, by investigating if they influence the RAAS.

There are few published studies explaining the influence of polyphenols and their metabolites on enzymatic inhibition of the RAAS, especially ACE and renin. Consequently we do not fully understand the mechanisms of polyphenolic action on the RAAS [366, 516-518]. Furthermore, those studies which have been undertaken on the modulation of ACE activity have generally incorporated polyphenol interventions at high concentrations, considerably above any typical or even moderately enhanced dietary intake, so their physiological relevance is limited. To generate more informative results regarding ACE and renin inhibition and the physiological effects of their modulation in cells, we used interventions comprising physiologically realistic plasma concentration post intakes in our *in vitro* experiments.

We began with trials of the *in vitro* effects of a range of common phenolic compounds on the RAAS at increasing concentrations. We explored their effects on renin gene expression in juxtaglomerular cells (AS4.1). Renin gene expression was significantly decreased following exposure to the oat polyphenols AV-B and t-FA, as well as several others present in plant products. t-FA possessed the strongest renin inhibitory activity, with a mean IC_{50} of 0.51 μM, the proportion of inhibition varied from 6.43 - 79.81 %. Other polyphenols from different food sources have previously been shown to modulate the RAAS system, by inhibiting renin *in vitro*. Renin suppression may have been due to the presence of polyphenol derived special functional structures such as analogues of galloyl moiety without a catechin skeleton and ortho-tri-hydroxy phenyl. Polyphenols may conform the renin active site, reducing its activity, by forming an enzyme-substrate-inhibitor complex. However, it is also possible that the complex biochemical transformations that the numerous polyphenols in plants, for example those in tea, undergo during the fermentation process which is typically part of its preparation for consumption, form a variety of bioactive substances, including *inter alia* theasinensin B, theasinensin C and a hexose conjugate. Previous studies have reported that polyphenols, especially gallated flavonoids, are able to inhibit renin activity, however, there is no strong evidence in the literature that any phenolics inhibit renin activity.

We went on to show that renin gene expression was significantly decreased following exposure to plant polyphenols via a mechanism that involved inhibition of the phosphorylation/activation of ERK1/2 and linked transcription factors CREB-1 and ATF-1. Several studies have shown that polyphenols influence ERK and Protein Kinase B (Akt) signalling pathways [556, 557]. For example, flavonols and anthocyanins in blueberry modulate the ERK-CREB-BDNF pathway in rodents [558]. In another study, quercetin and its O-methylated metabolites have been shown to induce neuronal death by inhibition of neuronal survival signalling through inhibition of ERK instead of activating the c-Jun N-terminal kinase-mediated death pathway [556]. There is also substantial evidence that the phosphorylation of CREB and its subsequent interference with the CRE, activates renin gene

transcription in juxtaglomerular cells, include AS4.1 and Calu-6 cells [509, 512, 515, 559-561]. In conclusion, it is widely reported that polyphenols interact with the ERK-ATF-1 pathway and P38/ CREB, to reduce expression of the renin gene, and that this leads to improved cardiovascular health [558, 562, 563].

We then explored the effects of selected polyphenols on ACE and NO using HUVEC cells *in vitro* and found most inhibited ACE, but to different degrees and none was significant; t-FA again showed the strongest inhibitory effect. ACE inhibition by tea polyphenols has been demonstrated in similar cell strains. ACE activity, and substrate-dependent reaction kinetics have revealed enzyme velocity curves that matched allosteric and non-Michaelis-Menten relationships, with a mixed mode of *in vitro* inhibition of ACE, mostly of a kinetically uncompetitive type [362], by green and black tea polyphenols. Further, studies on cultured endothelial cells from human umbilical veins incubated with a bilberry polyphenol extract exhibited significant, also inhibited ACE activity after 10 minutes, dose dependently [369]. However, unlike the studies in the literature; we failed to show experimentally that phenolic compounds significantly inhibit ACE protein levels and activity, and an increased production of nitric oxide. By suppression of renin and ACE, it was speculated that oat phenolics may be effective at reducing BP and thus CVD risk. There have been many previous studies that have reported the ACE inhibitory activity of a range of polyphenols [361, 362, 369], however, many of these have used polyphenols at concentrations much higher than that known to be present in the circulation following intake. Thus, these *in vitro* findings are not necessarily translatable to clinical or public health.

Our in-vitro findings suggest that these compounds possibly mediate changes in blood pressure through the RAAS system. However, bioavailability differs greatly from one polyphenol to another, so that the most abundant polyphenols in our diet may not necessarily be leading to the highest concentrations of active metabolites in target tissues. The metabolites present in blood, resulting from digestive and hepatic activity, usually differ from native compounds [693].

Since these results were promising, by clearly demonstrating that small phenolic compounds in physiological concentrations might affect the RAAS, albeit minimally, they may explain why people who consume foods rich in such polyphenols can experience a reduction in BP. Therefore, we conducted two human intervention trials using oat polyphenols at realistic dietary dosages, to expand on these findings and provide a better understanding of the physiological effects of specific polyphenols through both acute and chronic clinical responses.

5.3 HUMAN INTERVENTION TRIALS WITH OAT POLYPHENOLS

Epidemiological studies have suggested that high dietary intake of fruits and vegetables, as well as whole grains, is strongly associated with a reduced risk of developing chronic diseases such as cancer and cardiovascular disease [582, 587, 694]. Human intervention studies, to date have predominantly investigated the intake of whole grain oats, rather than the intake of individual oat bioactives, such as vitamins and minerals and/or phenolic components. Furthermore, many believe it is the fibre and phytochemical components of cereal grains that are the major contributing factors to their health benefits [40, 406, 588]. With respect to phytochemicals, phenolic acids such as hydroxycinnamic acid derivatives, including ferulic acid, *p*-coumaric acid and caffeic acid, as well as benzoic acid derivatives such as protocatechuic, vanillic, and syringic acids, are the predominant compounds. A meta-analysis of seven large cohort studies has found consumption of 2.5 compared to 0.2 servings of whole grain per day is associated with a 21 % decreased risk of CVD. As a result, a number of guidelines were drawn up with the aim of increasing intake of whole grain [695]. Consumption of 3 portions of whole-grain foods can significantly reduce cardiovascular disease risk in middle-aged people, mainly through blood pressure–lowering mechanisms. Decreases in systolic blood pressure are considered to reduce the incidence of coronary artery disease and stroke by ≥ 15 % and 25 %, respectively [597], whilst improved vascular responses and endothelial function has been associated with a daily consumption of wholegrain oat cereal [486].

A few human studies have focused on absorption and metabolism after consumption of whole grain wheat. Maximum hydroxycinnamate absorption from high-bran cereal, a particularly rich source of FA and diferulates because they include the outer husk including the aleurone layer, occurs between one and three hours after ingestion [455]. It was not found necessarily to be the case, though, that the main cleavage and release of FA from AX happens in the small intestine, but more likely that FA observed in plasma derived from the 4 % of free FA that the grain also contains. It also found low hydroxycinnamic acid levels in plasma after 6 hours, which suggests not much absorption occurring in the large intestine. This may be the result of long, slow FA cleavage as food passes through the large intestine, with minimal amounts being observed in the circulation. No diferulic acids were detected in urine or plasma and were most likely still in bound form, which would suggest that very little of these compounds was absorbed all the way through the gastrointestinal tract. All of this suggests the need for further human studies to fully understand absorption and metabolism of whole grain phenolic acids.

To date, however, there have been relatively few human trials conducted to assess the beneficial effects of oat polyphenols on the cardiovascular system and the potential mechanism of action. The

objective of our human intervention trials was, therefore, to assess the impact of consuming whole grain oats on cardiovascular health markers and in particular to discern the degree to which avenanthramides and phenolic acids may contribute to any physiological effects observed on human vascular function in healthy pre-or stage1 hypertensive individuals.

Our *in vitro* findings were not borne out in our acute clinical trial, which showed no statistically significant change in % FMD or BP following a single intake of oats, although we tentatively, given the limitations of the trial discussed above, propose that an increase endothelium-independent vasodilation occurred at early time points. The non-significant FMD and BP results compared to previous significant results of cocoa, berry and coffee could be due to: 1) the lower levels of phenolic intake delivered by the oats in our trial compared to those with, for example, coffee (310 mg compared to 50 mg for oat) [577]; 2) the small population size of our trial and consequent lack of statistical power, an issue discussed at length by Thies *et al*. (2014), although it was not significantly smaller than that used in some cocoa and blueberry clinical trials; or 3) the likelihood that other polyphenol interventions deliver more biologically active components, such as flavonoids, as has been widely reported [186, 255, 335, 581, 591].

In comparison to the acute trial, our chronic trial showed more positive evidence that the intake of phenolic acid rich oats may induce vascular improvements that are in terms of magnitude, possibly medically relevant. However, they generally failed to achieve statistical significance and the trial may have been undertaken with insufficient statistical power to draw more than indicative conclusions from secondary outcomes. Improvements were putatively noted for day- and night-time SBP and in vascular reactivity measured by FMD and LDI, following the intake of the highest phenolic acid containing oats for four weeks relative to the control intake. The failure to achieve statistically significant results in vascular function could be due to the small population size and the natural high variations in volunteer vascular measurements, such as BP and FMD which is indicated by large SEM in several results. Alternatively, as with the acute intervention, the nature and quantity of oat polyphenols delivered in our interventions may not be as biologically active as those used in published clinical trials, for example, flavanols in cocoa [44] and tea [681-683], with an intake of 100-200 mg/day having been shown to improve FMD and reduce 24 blood pressure [48, 286]. In addition, TC, HDL and LDL levels were not significantly lowered in our volunteers after four weeks, whereas 12 weeks consumption of cocoa powder (total flavanol 172 mg/day) has been shown to increase HDL cholesterol [640]. Also, oat intake has been found to significantly reduce TC in patient with hypercholesterolemia, whereas our population had only slightly elevated cholesterol levels [667]. Similarly, the intake of 40 g oats per day for 90 days was sufficient to significantly lower LDL

cholesterol in obese subjects [696, 697]. These differences in outcomes may also be due to the length of the trial, subject selection, and population size.

5.4 CONCLUSIONS

In our clinical trials we did not observe the same level of inhibition on renin or ACE as seen in our *in vitro* experiments. One potential reason for this was that the absorption of the oat phenolic acids and their metabolites is likely lower than that used in cell studies, i.e. below 1 µM. It is of limited value to predict the *in vivo* efficiency polyphenols based on cell trials, since numerous factors may affect *in vivo bioactivity*, including bioavailability, metabolism and tissue distribution. However, our *in vitro* observations make a positive contribution to establishing the role of oats phenolics as potential modulators of RAAS if sufficient concentrations are achieved in tissues (blood, endothelial and kidney). Both acute and chronic findings, need confirmation and clarification through further, expanded trials and it is likely, both from our own results and the literature, that the size of our trial and associated limitations, including the inherent variation in BP measures and other markers, the length of trial and the amount of the intervention, may all have contributed to the inconclusive outcomes.

The results of our work contribute to and extend existing knowledge of the effects of oat polyphenols on cardiovascular health. Our study is one of the first to address the possible relationships between oat polyphenols and the RAAS pathway and in so doing contributes important information in an area where knowledge is incomplete. Our results show evidence that the RAAS pathway is affected by phenolic oats and this may be an important physiological impact to add to others which are already more widely associated with cardiovascular health.

5.5 FUTURE PERSPECTIVES

Our findings clearly evidence the need for future studies to establish in detail the molecular mechanisms and to better quantify the clinical benefits of oat avenanthramides and phenolic acid. There is limited conclusive clinical data on the benefits of oat polyphenols and this study, while contributing and highlighting the potential for benefits, was also inconclusive. Hence further trials are needed to clarify effective dosage regimens to obtain optimal results. More extensive trials are also needed to understand optimal combinations of polyphenols, individual studies of fibre foods which contain polyphenols or high dose purified active polyphenol compounds from oats rather than the whole grain products that have been widely promoted and studied [40, 641]. Dosages, and the influences of mid- and long-term consumption on people with a range of cardiovascular markers, volunteer's diet history, all of which we shed light on but inconclusively, also require trials of

sufficient breadth and extent to be fully informative. Future trials should also be statistically powered to adequately interpret all the outcomes under consideration, this may mean very large trials, over 60 subjects, measuring a range of risk markers, or several smaller trials, with over 30 subjects in the intervention groups [40], considering just one or a small number of markers. The length of the trial is also critical, to adequately consider changes in RAAS enzyme levels and renin gene expression and to fully assess changes in BP related cardiovascular risk markers following polyphenol interventions. Previously published trials generally range from three to eight weeks, and several achieved statistically significant results only after eight weeks of trials [294, 299, 687, 688]. There is a lack of published information on how widely and evenly polyphenols are distributed in the body's tissues, their affinity for particular tissues and whether or not they are antagonised by reactions with other foodstuffs or cellular products which may alter their function, all of which should be the subjects of further study, since our 4 weeks chronic results showed only non-significant vascular improvements and blood pressure reduction. There are also the influences of genetic influence and variations to be considered. It is clear that the biochemical structure of the polyphenol affects its bioactivity and further research is needed to understand, not only which metabolites are most active in vascular function improvement but also which are most efficacious at realistic dietary intake levels.

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Appendix 1: PARTICIPANT INFORMATION SHEET

PARTICIPANT INFORMATION SHEET

Study title: PRO-GRAIN 2 (**P**henolic-**r**ich **o**ats for **g**ut-bacteria **r**elief and **a**rtery **i**mprovemen**t**)

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

Thank you for your interest in this study and taking your time to reading this participant information sheet.

Chief investigator: Professor Jeremy Spencer

Investigators: Dr Manuel Schar

Sarah Alsharif (PhD Student)

Angelika Kristek (PhD Student)

Gulten Soycan (PhD Student)

Patrizia Stanienda (MSc Student)

Study e-mail: pro.grain@reading.ac.uk

Telephone number: 0118 378 6833

Background

It is well known that what we eat and drink can have a major role in protecting us from disease. Eating more whole grain products can improve our health and this could, in part, be due to natural elements in whole grains such as phenolic acids. Indeed, regular intakes of phenolic acid related elements found in fruits, vegetables, wine, tea and chocolate has been shown to reduce our risk of suffering from cardiovascular disease. However, the health benefits of phenolic acids from wholegrains remains unclear.

What is the purpose of the study?

The main purpose of this study is to test if intake of a single portion of whole grain oats improves your heart and blood vessels health and if phenolic acids are, at least in part, responsible for these effects. Potential health benefits will be measured using a range of non-invasive and harmless tests. These include blood vessel elasticity, blood pressure, brain vessel function and potential further markers related to the cardiovascular system.

Am I suitable to take part?

We aim to recruit male subjects who are generally healthy with high normal to moderately elevated blood pressure. You need to be between 25 and 65 years of age, non-smoking, free of disease, not undertaking regular vigorous exercise and not taking long-term blood pressure or blood fat medication. At the beginning of your study participation, you will receive a health check and we will fully assess your eligibility to take part in the study.

Do I have to take part?

It is up to you to decide whether you wish to participate in the study. If you decide to proceed, you are free to withdraw at any time, without giving a reason. At your first visit, we will also describe the study to you in further detail and you can ask remaining questions. We will then ask you to sign a consent form to show you have agreed to take part.

What will happen if I decide to take part?

‘Health and lifestyle questionnaire’

If you wish to take part, please contact us and we will ask you to fill in a health and lifestyle questionnaire. Afterwards and if you meet our broad eligibility criteria, we will invite you to a consent and health check visit.

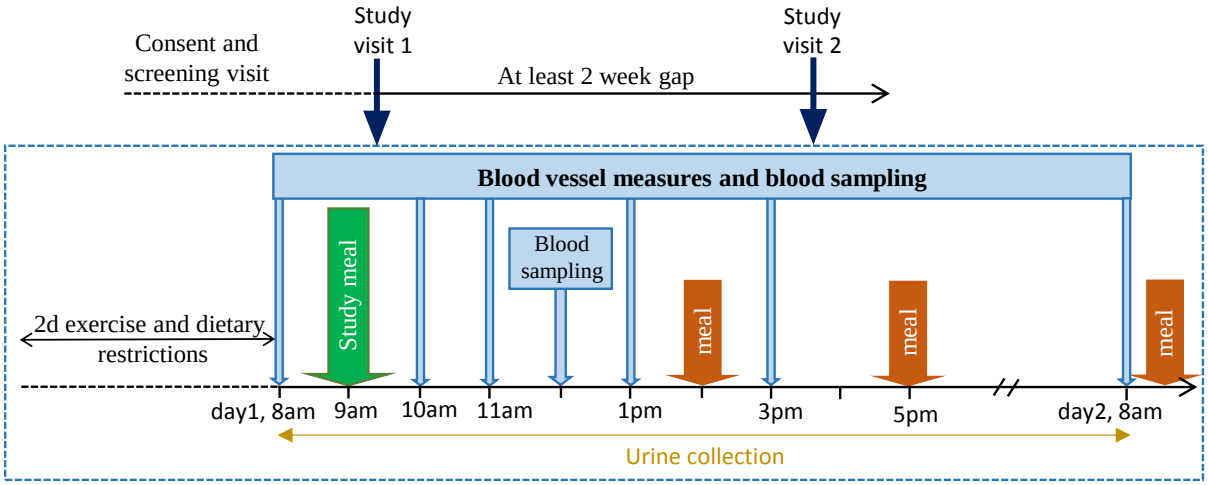
‘Consent and health check visit'

For a maximum 1 h morning visit, you will be asked to come to the Hugh Sinclair Unit of Human Nutrition in the Department of Food and Nutritional Sciences (University of Reading) in an unfed state (fasted, not eating or drinking anything but water from 8pm the night before). At the start of the visit, we will discuss the study, answer all your questions and you will then be asked to give consent for participating in this study. After that, we will measure your weight, height, blood pressure and a skilled nurse or phlebotomist will take a small blood sample (one tablespoon). You will be asked to fill in a questionnaire related to your habitual diet and we ask you to maintain your habitual diet during your study participation. You will receive a breakfast at the end of the visit.

After approx. 2-3 weeks, you will be informed of the results of your health check and whether or not you are suitable to take part in the study. If your results are outside the normal range, we will encourage you to go to your GP to discuss the results of your health check.

‘Study participation’

If you decide to take part and are suitable for the study, we will arrange 2 study visits at least 2 week apart from each other (See figure below).



On each study visit, we will ask you to stay with us at the Hugh Sinclair Unit of Human Nutrition from around 8am to 4pm and come back for a short follow-up visit the next morning. The visit will start with a questionnaire, measurement of your weight, height and body composition. A qualified nurse will then place a small flexible cannula into a vein in your arm, and this will remain in place with minimal discomfort to allow us to take blood samples during the study day.

You will then be asked to lie on a bed in a clinical room and we will perform a range of tests (blood vessel function, blood pressure etc.) that are all harmless and non-invasive, and collect small blood samples. Afterwards, you will be asked to consume a study meal, and subsequently the tests as well as blood sample collections will be repeated several times throughout the day (see figure above). We will also ask you to collect your urine until the end of the study visit. To ensure your well-being, we will only take little blood compared to a blood donor session and we will provide you plenty of water, a lunch (consisting of a cheese sandwich) and a dinner “to go” (consisting of a pasta bake and crème brulee). To get valid study results, it is vital that you do not eat and drink anything during the study visit duration, apart from the meals and water that we provide. This includes your time outside the unit during which you will be asked to only consume the dinner “to go” at a specified time (approx. 5pm) and nothing else but water. You will be asked to remain in the nutrition unit for the duration of study visit day 1 where you will have free access to water and facilities to watch TV, DVDs, work, read and use the internet. You will receive a breakfast at the end of study visit day 2.

Confidentiality, storage and disposal of information

Confidential information will be stored securely and can only be accessed by the study investigators. All information collected during the study will be treated in strict confidence in accordance with the relevant data protection legislation. No information will be disclosed in any way that will allow identification of yourself.

Are there any benefits/risks to taking part [e.g. health]?

An experienced nurse will perform the blood cannulation, while fasting blood sample collections will be taken by qualified and experienced phlebotomists. There may be a small amount of discomfort during the clinical measures. Although, the measures and blood-taking are generally safe and harmless, minor bruising may occur from the venipuncture or pressure applied by blood pressure

cuffs. Risk of allergies may also be possible; however, this risk will be kept to a minimum because only latex-free materials and low allergy plasters will be used.

What expenses and/or payment or equivalent will be made for participation in the study?

As an inconvenience allowance for your time and travel costs that you may incur, you will receive £200 for completing the study. In case of early withdrawal, you will receive the payment correspondent to the study visits you have completed.

What will the results of the study be used for?

The results of this study will be used in thesis of PhD and MSc students, will be published in a scientific journal and presented at international conferences.

Who has reviewed the study?

This project has been reviewed by the University of Reading Research Ethics Committee (Tel: +44 (0) 118 378 7119; Email: m.j.proven@reading.ac.uk) and has been given a favourable opinion for conduct.

Thank you for reading this information. Please contact us if you have any further questions or would like to take part in the study.

Appendix 2: Health and Lifestyle Questionnaire

All the information provided will remain confidential at all times.

Please fill the questionnaire below.

	DAY		MONTH			YEAR			
Date questionnaire completed									
Example	1	0	J	U	N	2	0	1	4

Personal Details

Title: Forename(s): Surname:		Address: Postcode:	
Daytime telephone no:		Evening telephone no:	
Mobile telephone no:		E-mail address:	
Best method (e.g. landline, mobile, e-mail) and time to contact you:			
Date of Birth:	Age today	Sex: Weight (kg): Height (m):	Proceed? Yes / No

Medical & Lifestyle Questions

Medical & Lifestyle Questions	Circle as appropriate:	Further Details:
Have you taken any antibiotics within the last 3 months?	YES NO	
Have you recently received any form of vaccination ?	YES NO	
Do you suffer from allergies to any food?	YES NO	
Are you currently taking part in another study ?	YES NO	
Have you recently taken part in a research study ? If YES, please give the study or researcher's name so we can contact them.	YES NO	
Do you smoke ?	YES NO	
If you are an ex-smoker, when did you stop?	Month: ..... Year:	
How much exercise do you participate in per week (not including walking)? Please specify: i) type of exercise(this includes cycling to work), ii) how often & iii) duration	YES NO	

Brief Medical History

Please tell us if you have any pre-existing health problems,including any of the following:

Medical Questions	Circle as appropriate:	Further Details:
Has your doctor told you that you suffer from heart disease (e.g. Angina), stroke or any other disease of the circulation (i.e. Reynaud's disease)?	YES NO	
Has your doctor diagnosed you as having high blood pressure (hypertension)?	YES NO	
Has your doctor diagnosed you as having high cholesterol?	YES NO	
Has your doctor diagnosed you as having diabetes (either type 1 (insulin dependent) or type 2 (age-onset diabetes))?	YES NO	
Has your doctor diagnosed you with cancer?	YES NO	
Has your doctor diagnosed you with HIV?	YES NO	
Do you have any other health problems?	YES NO	
Do you take any prescription medication?	YES NO	
Do you take any pain killing medication (e.g. aspirin, paracetamol, nurofen, ibuprofen etc) daily or more than 4 times per week ?	YES NO	

Dietary Intake and Supplement Use

Dietary Questions	Circle as appropriate:	Further Details:
Are you a vegan or vegeterian?	YES NO	
Do you take any form of dietary supplements? For example, minerals/vitamins, antioxidants, phytochemicals or fish oil supplements. If so, please give details and state the product name.	YES NO	
Do you drink alcohol ? If yes, how many units do you roughly drink per week ? A unit of alcohol is half a pint of beer/lager, a single pub measure of spirits e.g. gin/vodka or a small glass of wine(125ml).	YES NO	

Thank you for completing this questionnaire.

Appendix 3: CONSENT FORM

Consent Form

Initials

1. I have read and had explained to me by …….. the accompanying Information Sheet relating to the PROGRAIN 2 study ☐

I have had explained to me the purposes of the project and what will be required of me, and any questions I have had have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation. ☐

I understand that participation is entirely voluntary and that I have the right to withdraw from the project any time, and that this will be without detriment. ☐

I authorise the Investigator to consult my General Practitioner, and provide their name and address details overleaf. ☐

This application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct. ☐

I have received a copy of this Consent Form and of the accompanying Information Sheet. ☐

I consent to my contact details being stored on the Hugh Sinclair Unit of Human Nutrition Volunteer Database. ☐

I consent to my screening information (date of birth, height, weight, blood pressure smoking status, long-term use of medication, and blood test results such as levels of cholesterol, liver enzymes, haemoglobin, blood cell count) being stored on the Hugh Sinclair Unit of Human Nutrition Volunteer Database. ☐

Yes ☐ No ☐

Name: ……

Date of birth: ……
Signed: ……

Date: ……

GP details

Name: ……

Address: ……

……

……

Witnessed by: Name……Signature……Date……

Appendix 4: Preparation Instructions

Instant Oatmeal:

1. Take pre-weighted oats out of the freezer and give 5min to bring back to room temperature.
2. Pour content in a bowl
3. Add 420 g of boiling water (determine boiling temperature and consistently reach this temperature each time); stir
4. Let sit for 1 minute before serving

Cream of Rice:

1. Take container with pre-weighted cream of rice, dry skimmed milk and pectin powder out of the freezer and give 5 min to bring back to room temperature.
2. Pour content in a bowl
3. Add 6.1g of sunflower oil.
4. Add 250 g of boiling water (determine boiling temperature and consistently reach this temperature each time); stir
5. Let sit for 1 minute before serving
6. Serve with 170g water

Cream of Rice will be purchased from B&G Foods, Inc.

Appendix 5: Dietary restrictions in preparation for the study

To make sure that our measurements at your visit show valid results, it is very important that you avoid any foods that contain phenolic acids for 2 days prior to the study day.

Foods to avoid	Foods that can be eaten
☹ Oats, porridge, or oat bars ☹ Whole wheat bread, cereal or products ☹ Products containing other whole grains (rye, barley or spelt) ☹ Fruits ☹ Vegetables ☹ Soups ☹ Spices ☹ Herbs ☹ Flavourings ☹ Nuts and seeds ☹ Chocolate ☹ Crisps flavoured with spices ☹ Jams, chutneys and dips	☺ White bread (plain, no whole grain, without seeds) ☺ Cereal (plain, no whole grain, without fruit, seeds or nuts etc.) ☺ Meat ☺ Fish ☺ Poultry ☺ Eggs ☺ Pasta (plain) ☺ Rice (plain) ☺ Potatoes ☺ Plain crisps (ready salted, salt & vinegar) ☺ Butter and margarine ☺ Dairy (plain cheese without fruit or spices/hers. Vanilla or greek yoghurts only)

Beverages to avoid	Beverages that can be consumed
☹ Fruit juices ☹ Squash/cordial ☹ Tea including herbal tea ☹ Coffee ☹ Caffeinated drinks (e.g. red bull, coke) ☹ Hot chocolate ☹ Alcohol (wine, beer, spirits etc.) ☹ Soya, almond or oat milk	☺ Water ☺ Dairy Milk

Meal examples:

✓ Eggs and ham ✓ Macaroni cheese ✓ Fish & chips ✓ Baked potatoe with tuna	✓ Ham & cheese sandwich ✓ Egg mayonnaise sandwich ✓ Cheese omelette ✓ Mince and potatoes

University of Reading | HUGH SINCLAIR Unit of Human Nutrition

Appendix 6: PARTICIPANT INFORMATION SHEET

DevOAT study: DevOAT yourself to a healthy lifestyle

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully and discuss it with family and friends if you wish. Ask us if there is anything that is not clear or if you would like more information.

Thank you for your interest in this study and taking your time to read this participant information sheet.

Chief investigator: Professor Jeremy Spencer

Investigators: Dr Manuel Schar

Sarah Alsharif (PhD Student)

Angelika Kristek (PhD Student)

Gulten Soycan (PhD Student)

Hanna Petsch (MSc Student)

Study e-mail: DevOAT.study@reading.ac.uk Telephone number: 0118 378 6833

Background

It is well known that what we eat and drink can have a major role in protecting us from disease. Eating more whole grain products can improve our health and this could, in part, be due to natural elements in whole grains such as phenolic acids. Indeed, regular intakes of phenolic acid related elements found in fruits, vegetables, wine, tea and chocolate have been shown to reduce our risk of suffering from cardiovascular disease. Fibre from whole grains, on the other hand, is an indigestible carbohydrate (that is usually part of plant foods) with many health effects, mainly by feeding the good bacteria in our gut and easing bowel movements. However, the health benefits of phenolic acids from whole grains remain unclear.

What is the purpose of the study?

The main purpose of this study is to test if long-term intake of a 70g breakfast cereals and 50g snacks improves your heart, blood vessels and intestinal health; and if phenolic acids are, at least in part, responsible for these effects. Potential health benefits will be measured using a range of non-invasive and harmless tests. These include blood vessel elasticity, blood pressure, gut-bacteria and further markers related to the cardiovascular system.

Am I suitable to take part?

We aim to recruit people who are generally healthy within study range above 120/75 and 160/100 mmHg. You need to be between 25 and 75 years of age, non-smoking (or who stopped smoking in last 3 month), free of disease, not undertaking regular vigorous exercise and not taking long-term blood pressure or blood fat medication.

Females taking the contraceptive pill or hormone replacement therapy (HRT) can take part. Females who are pregnant, lactating or, if of reproductive age and not using a reliable form of contraception (including abstinence) will not be able to take part in the study.

At the beginning of your study participation, you will receive a health check and we will fully assess your eligibility to take part in the study.

Do I have to take part?

It is up to you to decide whether you wish to participate in the study. If you decide to proceed, you are still free to withdraw from the study at any time, without giving a reason. At your first visit, we will also describe the study to you in further detail and you can ask remaining questions. We will then ask you to sign a consent form to show you have agreed to take part.

What will happen if I decide to take part?

‘Health and lifestyle questionnaire’

If you wish to take part, please contact us and we will ask you to fill in a health and lifestyle questionnaire. Afterwards and if you meet our broad eligibility criteria, we will invite you to a consent and health check visit. The consent will be taken in an unfed state (fasted, not eating or drinking anything but water from 8pm the night before).

‘Consent and health check visit'

For a maximum 1 h morning visit, you will be asked to come to the Hugh Sinclair Unit of Human Nutrition in the Department of Food and Nutritional Sciences (University of Reading) in

an unfed state (fasted, not eating or drinking anything but water from 8pm the night before). At the start of the visit, we will discuss the study, answer all your questions and you will then be asked to give consent for participating in this study.

After that, we will measure your weight, height, blood pressure and a skilled nurse or phlebotomist will take a small blood sample (equivalent to two tablespoons). You will be asked to fill in a questionnaire related to your habitual diet and we ask you to maintain your habitual diet during your study participation. You will receive a breakfast at the end of the visit.

After approx. 2-3 weeks, you will be informed of the results of your health check and whether or not you are suitable to take part in the study. If your results are outside the normal range, we will ask for your consent to send the results to your GP and encourage you to discuss these with your GP. If you are eligible we will inform your GP that you volunteered to participate in our study.

'Familiarisation visit'

If you decide to take part and are suitable for the study, we will arrange a familiarisation visit at the Hugh Sinclair Unit of Human Nutrition. A demonstration of the vascular measurements will be given, while all further aspects of the study will be discussed in detail, including the gastrointestinal questionnaires, 3 day food diaries, intake of the study intervention materials, collection of the stool and urine samples and dietary & exercise restrictions.

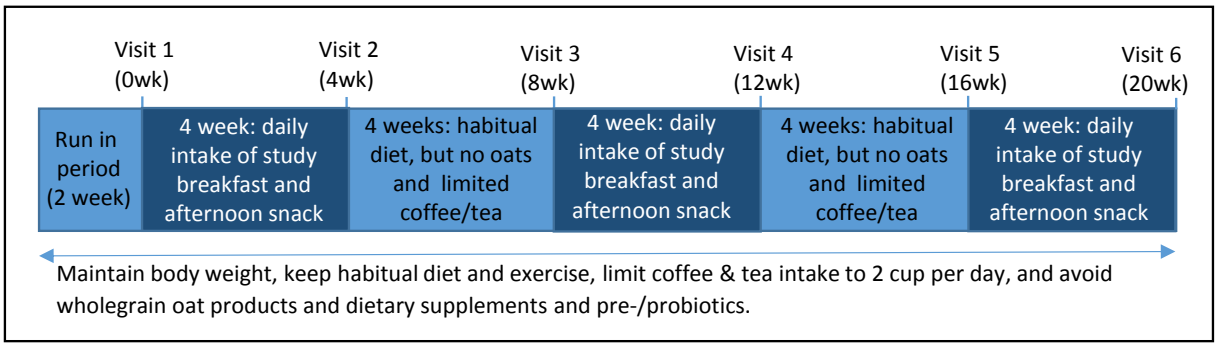
`Dietary restrictions`

For a two week period prior to your first study visit and during your total study participation (22 weeks), you will be asked to **follow your habitual diet but to limit your intake of phenolic acid rich beverages (i.e. coffee and tea) to no more than 2 cups (400ml) per day and not to consume confirmed probiotics** (e.g. live yoghurts, fermented milk drinks), supplementary **prebiotics** (such as inulin) (check last page of the Information sheet) and avoid **any oat products**.

`Study participation`

If you decide to take part and are suitable for the study, we will contact you in order to offer you an option of appointments for your study participation lasting 22 weeks in total (see figure below). Your participation will include that on three periods lasting 4 weeks each you eat a specific breakfast and afternoon snack every day, and that you attend six study visits at the Nutrition Unit. The day after each of these six visits, we will also ask you to drop off the blood pressure monitor, urine and stool samples (5min visit). Overall there are 14 visits to the Nutrition Unit (including health check and familiarisation visits). We will provide the breakfasts and afternoon snacks, which will consist of products made of oats, rice or wheat.

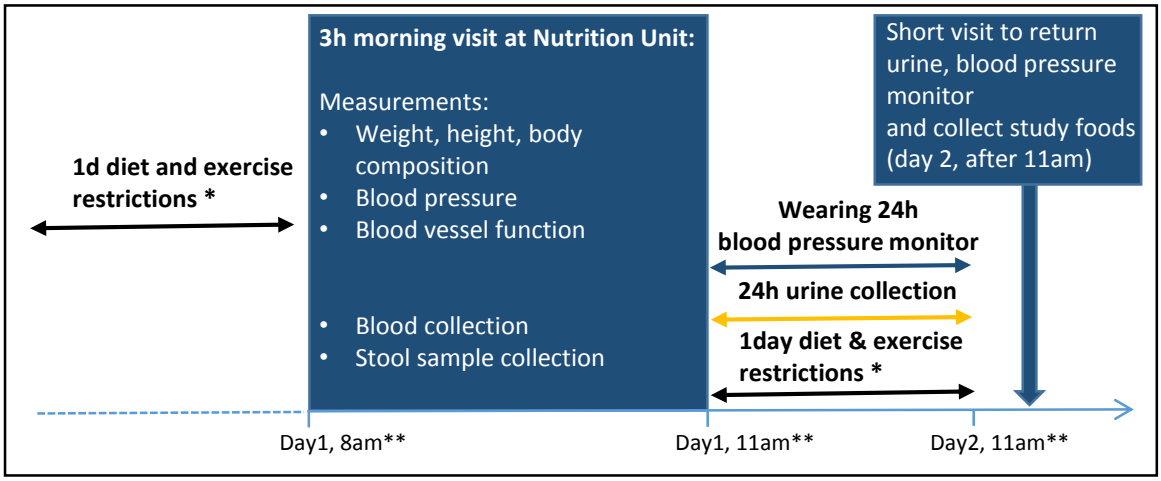
A Study overview



Study visits`

On each study visit, we will ask you to stay with us for 3 hours at the Hugh Sinclair Unit of Human Nutrition (for example from 8am to 11am) in an overnight fasted state (see figure below). The visit will start with a questionnaire, measurement of your weight, height and body composition. You will then be asked to lie on a bed in a clinical room and we will perform a range of tests (blood vessel function, blood pressure etc.) that are all harmless and non-invasive, and collect a small blood sample (3 tablespoon). Anytime during the course of the morning we will also require you to provide a stool sample. We will provide you with a breakfast before you leave the Nutrition Unit.

For the 24h after your visit, we will ask you to collect your urine and to wear a blood pressure monitor that will inflate every 30min during daytime and every hour during night-time. The following day, at a time convenient to you after 11am, we will ask you to return the urine and blood pressure monitor to us. Furthermore, you will be given the breakfasts and afternoon snacks, which you will be consuming over the following 4 weeks.



* Restrictions include: no strenuous exercise and no intake of a range of foods (see list on last page). On the evening before the visit, we will ask you to consume a provided dinner and then fast ≥12h.
** Indicated times are only an example and can be agreed on an individual basis.

Before each study visit, you will also be asked to complete a 28-day gastrointestinal well-being diary and a 3-day food diary. Moreover, from 24h before your study visit to the end of your blood pressure monitoring you will be asked to abstain from vigorous exercise and follow dietary restrictions low in phenolic acids, and free of alcohol, caffeine and fish (see list on last page).

Confidentiality, storage and disposal of information

Confidential information will be stored securely and can only be accessed by the study investigators. All information collected during the study will be treated in strict confidence in accordance with the relevant data protection legislation. No information will be disclosed in any way that will allow identification of yourself.

Are there any benefits/risks to taking part [e.g. health]?

By taking part in this study you will be helping us understand more about the health benefits of phenolic acids from whole grains.

The fasting blood sample collections will be taken by qualified and experienced phlebotomists or nurse. There may be a small amount of discomfort during the clinical measures. Although, the measures and blood-taking are generally safe and harmless, minor bruising may occur from the venepuncture or pressure applied by blood pressure cuffs. Risk of allergies may also be possible; however, this risk will be kept to a minimum because only latex-free materials and low allergy plasters will be used.

What if I do not wish to take part or change my mind?

The study is voluntary so that you should not feel under any pressure to take part. If you do decide to take part you are still free to withdraw at any time.

What if something goes wrong?

In the event that something does go wrong and you are harmed during the study the University of Reading has in place Professional Indemnity Insurances that provide cover against negligence, error or omission for the activities of its employers.

Who is sponsoring and funding the research

This study is sponsored by the University of Reading and financed by the UK government (90 %) and PepsiCo Research & Development (10 %).

What expenses and/or payment or equivalent will be made for participation in the study?

As an inconvenience allowance for your time and travel costs that you may incur, you will receive £300 for completing the study. In case of early withdrawal, you will receive the payment correspondent to the study days you have completed.

What will the results of the study be used for?

The results of this study will be used in the thesis of PhD and MSc students, will be published in a scientific journal and presented at international conferences.

Who has reviewed the study?

This project has been reviewed by the University of Reading Research Ethics Committee (Tel: +44 (0) 118 378 7119; Email: m.j.proven@reading.ac.uk) and has been given a favorable opinion for conduct.

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favourable opinion by the London – Bromley Research Ethics Committee.

Who do I contact for further information or complaints?

Main point of contact:	For formal complaints:
Dr Manuel Schar *Email:* DevOat.study@reading.ac.uk *Tel:* 0118 378 6833 *Address:* Department of Food and Nutritional Sciences, PO Box 266, University of Reading, Whiteknights Campus, Reading, RG6 6AP	Prof. Jeremy Spencer (Principal Investigator) *Email:* j.p.e.spencer@reading.ac.uk *Tel:* 0118 378 8724 *Address:* Department of Food and Nutritional Sciences, PO Box 266, University of Reading, Whiteknights Campus, Reading, RG6 6AP

Thank you for reading this information. Please contact us if you have any further questions or would like to take part in the study.

This study has been reviewed according to procedures specified by the University of Reading Research Ethics Committee and has been given a favourable opinion for conduct (www.reading.ac.uk/internal/res/ResearchEthics)

One copy to be given to the participant and one copy to be kept in the investigator study file.

Appendix 7: Medical and Lifestyle Questionnaire

Chronic cardiovascular and gut-bacteria effects of phenolic rich oats in adults with above average blood pressure.

Name:		Title:
Address:		Date of Birth: Age:
Daytime Telephone:	Evening Telephone:	Best time to call:
Weight (kg):	Height (m):	BMI (kg/m^2):
E-mail: Do you use emails on a regular basis? YES/NO		

How did you hear about the study? _____

Please circle as appropriate

Medical questions

Have you been diagnosed as having any of the following?

a) High blood cholesterol
YES/NO

b) High blood pressure
YES/NO

If you recently had your blood pressure measured, can you give us a rough estimate of what the pressure was? (Reference example: 120 / 80 mmHg)

c) Thyroid disorder YES/NO

d) Diabetes or other endocrine disorders YES/NO

e) Heart problems, stroke or any vascular disease in the past 12 months YES/NO

f) Inflammatory diseases (e.g. rheumatoid arthritis) YES/NO

g) Renal, gastrointestinal, respiratory or liver disease YES/NO

h) Cancer
YES/NO

i) Chronic constipation, diarrhoea or other chronic gastrointestinal complaint (e.g.

irritable bowel syndrome)
YES/NO

Have you been diagnosed as suffering from any other illness?
YES/NO

If 'YES', please give details

Within the past 3 months, have you taken any medication (prescription or non-prescription)?
YES/NO

If 'YES', what are they and for what reasons?

Have you had any surgery within the past 3 months or do you have surgery planned? YES/NO
If 'YES', please give details

Have you ever suffered from a pulmonary embolism, deep vein thrombosis, blood clots or had a blood transfusion?
YES/NO

If 'YES', please give details

Do you have a pacemaker? YES/NO

Do you regularly take painkillers? YES/NO

If 'YES', please give details

Lifestyle questions

Are you currently taking part in or within the last 3 months been involved in a clinical trial or a research study? YES/NO

If 'YES', please give details:

Have you been screened or contacted recently about a study? YES/NO

If 'YES', please give details

Are you a blood donor? YES/NO

If 'YES', when was the last time you gave blood?

If you are eligible to participate in the study, are you willing to postpone further blood donations until 3 months after your final study visit? YES/NO

Do you have any food allergies (e.g. gluten or dairy) or intolerances (e.g. lactose)?
YES/NO

If 'YES', what are they?

Do you use any of the following:

Dietary supplements, e.g. fish oils, evening primrose oil, vitamins or minerals (such as iron or calcium); YES/NO

Probiotics, e.g. Actimel, Yakult, Activia yoghurts or capsules; YES/NO

Cholesterol-lowering products, e.g. Flora Pro-Activ or Benecol? YES/NO

If 'YES' to any, please give details

Are you vegetarian or vegan? YES/NO

If 'YES', please specify

Are you following or planning to start a restricted diet, e.g. to lose weight? YES/NO

If 'YES', would you be willing to postpone this until after your final study visit? YES/NO

Do you drink alcohol? YES/NO

If 'YES', approximately how many units do you drink per week? ____Units

One unit of alcohol is half a pint of beer/lager, a single pub measure of spirits e.g. gin/vodka, or a small glass of wine (125 ml).

How much exercise do you participate in per week (not including walking)?

Please specify: i) type of exercise (this includes cycling to work), ii) how often & iii) duration

Do you regularly consume wholegrain (e.g. breakfast cereal, dark bread, brown rice)?

If so, please say which types and estimate how many servings per day.

Do you regularly consume coffee?

If so, please estimate how many cups per day.

Do you regularly consume tea?

If so, please estimate how many cups per day.

Do you smoke?
YES/NO

If 'YES, please give details

If female, are you pregnant, intending to become pregnant or breast-feeding? YES/NO

If female, are you using contraception?
YES/NO

If yes, please give details.

This is the end of the questionnaire - thank you for your time.

All information provided will remain confidential at all times.

Appendix 8: Consent Form

Study title: Devoat study (**P**henolic-**r**ich **o**ats for **g**ut-bacteria **r**elief and **a**rtery **i**mprovemen**t**)

Initials

1. I confirm that have read and had explained to me by …….. the accompanying Information Sheet relating to the DevOAT study ☐

I have had explained to me the purposes of the project and what will be required of me, and any questions I had were answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation. ☐

I understand that participation is entirely voluntary and that I have the right to withdraw from the project any time without giving any reason, and that this will be without detriment ☐

I understand that relevant sections of any of my data collected during the study, may be looked at by responsible individuals from University of Reading , where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records ☐

I authorise the Investigator to consult my General Practitioner, provide their name and address details overleaf and inform the GP about my study participation. ☐

I agree for my blood, urine, stool samples to be stored for future use in this research study ☐

This application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct. ☐

I have received a copy of this Consent Form and of the accompanying Information Sheet. ☐

I consent to my contact details being stored on the Hugh Sinclair Unit of Human Nutrition Volunteer Database. ☐

Initials

I consent to my screening information (date of birth, height, weight, blood pressure smoking status, long-term use of medication, and blood test results such as levels of cholesterol, liver enzymes, haemoglobin, blood cell count) being stored on the Hugh Sinclair Unit of Human Nutrition Volunteer Database. ☐

Name: ……

Date of birth: ……

Signed: ……

Date: ……

GP details

Name: ……

Address: ……

……

Witnessed by

Name……Signature…….

Date……

One copy to be given to the participant and one copy to be kept in the investigator study file.

Appendix 9: Dietary restrictions in preparation for the study

To make sure that our measurements at your visit show valid results, it is very important that you avoid any foods that contain phenolic acids for **1 day** prior to the six study days.

Foods to avoid	Foods that can be eaten
☹ Oats, porridge, or oat bars ☹ Whole wheat bread, cereal or products ☹ Products containing other whole grains (rye, barley or spelt) ☹ Fruits ☹ Vegetables ☹ Vegetable oils ☹ Soups ☹ Spices ☹ Herbs ☹ Flavourings ☹ Nuts and seeds ☹ Chocolate and chocolate biscuits ☹ Crisps flavoured with spices ☹ Jams, chutneys and dips ☹ Fish	☺ White bread (plain, no whole grain, without seeds) ☺ Cereal (plain, no whole grain, without fruit, seeds or nuts etc.) ☺ Meat ☺ Poultry ☺ Eggs ☺ Pasta (plain) ☺ Rice (plain) ☺ Potatoes ☺ Plain crisps (ready salted) ☺ Butter and margarine ☺ Plain cheese (without fruit or spices/herbs) ☺ Plain or Greek style yoghurts

Beverages to avoid	Beverages that can be consumed
☹ Fruit juices ☹ Squash/cordial ☹ Tea including herbal tea ☹ Coffee ☹ Caffeinated drinks (e.g. red bull, coke) ☹ Hot chocolate ☹ Alcohol (wine, beer, spirits etc.) ☹ Soya, almond or oat milk	☺ Water ☺ Dairy Milk
	Meal examples: ☺ Eggs and ham ☺ Macaroni cheese ☺ Baked potato with tuna ☺ Ham & cheese sandwich ☺ Egg mayonnaise sandwich ☺ Cheese omelette ☺ Mince and potatoes

Appendix 10: 24-HOURS AMBULATORY BLOOD PRESSURE MEASURMENT DIARY

Volunteer number: Diary number: 1/2/3/4/5/6

Day: M / Tu / W / Th / F / Sa / Su Date:

ABP Number :

Time you went to sleep: Time you woke up:

Did you do any exercise? Y / N If yes, at what time:

Note: you do not need to record your activities for the first two readings.

Time	Activity (please circle)			Other activity / comments
07:00	Lying down	Sitting	Standing	
07:30	Lying down	Sitting	Standing	
08:00	Lying down	Sitting	Standing	
08:30	Lying down	Sitting	Standing	
09:00	Lying down	Sitting	Standing	
09:30	Lying down	Sitting	Standing	
10:00	Lying down	Sitting	Standing	
10:30	Lying down	Sitting	Standing	
11:00	Lying down	Sitting	Standing	
11:30	Lying down	Sitting	Standing	
12:00	Lying down	Sitting	Standing	
12:30	Lying down	Sitting	Standing	
13:00	Lying down	Sitting	Standing	
13:30	Lying down	Sitting	Standing	
14:00	Lying down	Sitting	Standing	
14:30	Lying down	Sitting	Standing	
15:00	Lying down	Sitting	Standing	
15:30	Lying down	Sitting	Standing	
16:00	Lying down	Sitting	Standing	
16:30	Lying down	Sitting	Standing	

17:00	Lying down	Sitting	Standing	
17:30	Lying down	Sitting	Standing	
18:00	Lying down	Sitting	Standing	
18:30	Lying down	Sitting	Standing	

Time	Activity (please circle)			Other activity / comments
19:00	Lying down	Sitting	Standing	
19:30	Lying down	Sitting	Standing	
20:00	Lying down	Sitting	Standing	
20:30	Lying down	Sitting	Standing	
21:00	Lying down	Sitting	Standing	
21:30	Lying down	Sitting	Standing	
22:00	Lying down	Sitting	Standing	
23:00	Lying down	Sitting	Standing	
00:00	Lying down	Sitting	Standing	
01:00	Lying down	Sitting	Standing	
02:00	Lying down	Sitting	Standing	
03:00	Lying down	Sitting	Standing	
04:00	Lying down	Sitting	Standing	
05:00	Lying down	Sitting	Standing	
06:00	Lying down	Sitting	Standing	