University of Reading

‘Effects of Oat Phenolic Acids and Avenanthramides on Cardiovascular Health’

Thesis submitted for the Degree of

Doctor of Philosophy

Department of Food and Nutritional Sciences

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September 2019

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.

Gulten Soycan

Reading, September 2019

Acknowledgements

Firstly, I would like to thank my supervisor Professor Jeremy Spencer, without his support and guidance this journey would not have been possible. Thank you for believing my potential and giving me this life changing opportunity, which I have learnt a lot. Thanks also to my secondary supervisor Dr Giulia Corona for her valuable advice and support throughout my PhD and Professor Peter Shewry for his endless help during the project.

I would like to thank to Dr Manuel Schar for his endless support and patience, without his help, this experience would have been much more difficult. A special thanks to Angelika Kristek and Sarah Al Sharif for being very hard working and patient enough to carry out the demanding human intervention studies in a limited time.

I would like to thank everyone in the Hugh Sinclair Nutrition Unit, all members of the lab group, who supported me during my PhD. Moreover, I am thankful to the study volunteers for their interest in our research and their study participation.

Finally, an important thank you to my family and friends for their endless support and encouragement. Thank you for never leaving me alone and teaching me how to be strong when I needed it the most.

Abstract

High consumption of wholegrains is associated with lower risk of cardiovascular disease. Studies suggested that regular consumption of oats lowers blood cholesterol and improves post-prandial glycaemic control. Some of these benefits were shown to be partly mediated by β-glucan soluble fiber. However, oats are also a source of bioactive phenolics, including the most abundant ferulic acid and avenanthramides. Circulating phenolic acids from other foods/beverages such as blueberries and champagne were shown to exert health benefits via enhancing nitric oxide bioavailability to the vascular endothelium, research to date has not yet established whether oat phenolics may also contribute to cardiovascular health benefits of oats. Therefore, this thesis investigates the potential beneficial effects of oat phenolics on human cardiovascular system.

Composition and content of a variety of commercial oat products were analyzed and average amounts delivered to the consumer in a portion was estimated as follows: 15.79-25.05 mg /40 g for oat products and 16.7 mg /11 g for oat bran concentrate. While the compositions and concentrations of the components in the different products were broadly similar, most abundant phenolic acid was shown to be ferulic acid. The findings suggested that commercial oat products are a source of phenolic acids and avenanthramides and these data informed following clinical work about human dietary intake levels of these compounds in oats.

A 2-arm, randomized, crossover, single-blind acute clinical trial was conducted with 16 pre and stage 1 hypertensive men. Consumption of 90.2g of oats containing 50mg of phenolics resulted in a small non-significant improvement in flow mediated dilatation (FMD) at 1h and significant changes in both endothelium dependent and independent microvascular function relative to control at 2h. Inter-individual variability in phenolic absorption observed and changes in microvascular function at 2h were positively correlated with the blood concentration of phenolic acid metabolites including ferulic acid glucuronides and sulfates. However, no other changes in blood pressure (BP) and NADPH oxidase activity were observed between treatments.

In a 3-arm, randomized , crossover, double-blind chronic trial , 28 pre and stage 1 hypertensive subjects consumed either a high (68.1 mg of phenolics) or moderate (38.9 mg of phenolics) phenolic acid and avenanthramide containing oat diet, or fibre matched control diet. 24-hour SBP, night time SBP and DBP decreased significantly and FMD response improved non-significantly following the consumption of high phenolic oats. There was also small non-significant improvements in endothelial dependent and independent microvascular reactivity and non-significant reductions in total and LDL-cholesterol following the consumption of high and moderate phenolics but no change observed in the numbers of circulating extracellular vesicles. These data suggest that consumption of oat phenolics may improve cardiovascular health and strengthen the evidence about health benefits of oats.

List of Publications and Presentations

Publications

Soycan, G., Schär, M.Y., Kristek, A., Boberska, J., Alsharif, S. N., Corona, G., Shewry, P. R. and Spencer, J. P.,2019. Composition and content of phenolic acids and avenanthramides in commercial oat products: Are oats an important polyphenol source for consumers?. Food Chemistry: X,3 100047.

Kristek, A., Wiese,P., Heuer. P., Kosik, O., Schär, M.Y., Soycan, G., Alsharif, S. N., Kuhnle, G.G.,Walton, G., Spencer, J. P 2019 Oat bran, but not its isolated bioactive β-glucans or polyphenols, have a bifidogenic effect in an in vitro fermentation model of the gut microbiota British Nutrition Journal-121(5)-549-559

Kristek, A., Schär, M.Y., Soycan, G., Alsharif, S. N., Kuhnle, G.G.,Walton, G., Spencer, J. P 2018 The gut microbiota and cardiovascular health benefits: A focus on wholegrain oats Nutrition Bulletin – 43(4),358-373.

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.

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

Chronic effects of oat phenolic acids and avenanthramides in pre or stage-1 hypertensive adults.
Gulten Soycan, Manuel Schär, Angelika Kristek, Sarah Alsharif, Giulia Corona, Peter Shewry Jeremy Spencer
13th European Nutrition Conference , FENS 2019, Dublin, Ireland. 15th-18th of October 2019

Chronic effects of oat phenolic acids and avenanthramides in pre or stage-1 hypertensive adults
Gulten Soycan, Manuel Schär, Angelika Kristek, Sarah Alsharif, Peter Shewry, Jeremy Spencer
The 2nd International Conference on Food Bioactives and Health. Lisbon, Portugal from 26th to 28th September 2018.

Identification and Quantification of Phenolic acids in Commercial Oat Varieties
Gulten Soycan Manuel Schär, Angelika Kristek, Sarah Alsharif, Giulia Corona, Peter Shewry Jeremy Spencer.
8th International Conference on Polyphenols and Health. Québec City, Canada From October 3 to 6, 2017.

Effects of oat phenolic acids on NADPH oxidase activity in pre and stage 1 hypertensive men
Gulten Soycan, Manuel Schär, Angelika Kristek, Sarah Alsharif, Giulia Corona, Peter Shewry Jeremy Spencer.
The 1st International Conference on Food Bioactives and Health. Norwich, UK from 13th to 15th September 2016.

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List of Abbreviations

°C	Degrees Celsius
µL	Microlitre
Ach	Acetylcholine
Alx	Augmentation Index
Akt	Protein Kinase B
AMBP	Ambulatory Blood Pressure
AMPK	Adenosine Monophosphate-activated Protein Kinase
AUC	Area under the curve
AV	Annexin V
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
DAD	Diode Array Detector
DBP	Diastolic Blood Pressure
EDEVs	Endothelial derived extracellularvesicles
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety and Authority
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
EVs	Extracellular Vesicles
FMD	Flow mediated dilatation
FMO	Fluorescence minus one
FW	Fresh weight
G	Gram
GTPase	Guanosin triophosphate
HDL	High-Density Lipoprotein
Hg	Mercury
HPLC	High performance liquid chromatography
HR	Heart Rate
HRT	Hormone Replacement Therapy
HUVEC	Human umbilical vein endothelial cells
iAUC	Incremental Area Under Curve
IHD	Ischaemic Heart Disease
Kcal	Kilocalorie
Kg	Kilogram
L	Litre
LDI	Laser Doppler Iontophoresis
LDL	Low-Density Lipoprotein
LPH	Lactase phloridzin hydrolase
MAECs	Mouse aortic endothelial cells
MAPK	Mitogen-Activated Protein Kinase
Mg	Milligram
ml	Millilitre
Mm	Millimolar
Na	Sodium
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NHS	National Health Service
nM	nanoMolar
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NOx	Nitrite/nitrate
ox-LDL	Oxidised Low-Density Lipoprotein
PCA	Principal Component Analysis
PDEVs	Platelet derived extracellular vesicles
PFP	Platelet poor plasma
PGI2	Prostacyclin
PMA	Phorbol myristate aceate
PP	Pulse Pressure
PS	Phosphatidylserine
PWA	Pulse Wave Analysis
PWV	Pulse Wave Velocity

RCT	Randomised control trial
ROS	Reactive Oxygen Species
RT	Room Temperature
SACN	Scientific Advisory Committee on Nutrition
SBP	Systolic Blood Pressure
SGLT-1	Sodium-dependent glucose transporter
sMRM	Scheduled multiple reaction monitoring
SNP	Sodium Nitroprusside
TAG	Triacylglycerol
TC	Total Cholesterol
TNFα	Tumour Necrosis Factor-Alpha
UPLC-MS/MS	Ultra performance liquid chromatography-Mass spectrometry
VCAM	Vascular cell adhesion molecules
VCAM-1	Vascular cell adhesion molecule 1

Chapter 1 Introduction

1.1 Overview

Cardiovascular disease (CVD) is the number one cause of mortality globally, accounting for 31% of all deaths (1). Of these deaths, coronary heart disease (CHD) and stroke are the most common forms and over three quarters of deaths take place in low and middle-income countries(1). CVD accounts for 45% and 25% of all deaths in Europe and the UK, respectively (2, 3). Although there are now treatments and rehabilitation available and the most recent published Global Burden of Disease figures showed a significant decline in the mortality rates, more than 4 million people still die from CVD in Europe every year (2, 4). Early detection and management of the disease through modifiable risk factors is fundamental and the focus should be on reduction of these risk factors by encouraging health lifestyle choices(1).

CVD have complex multifactorial aetiology underlying a number of non-modifiable and modifiable risk factors(5). Non-modifiable risk factors include age, ethnicity, gender, genetic predispositions and family history of premature CVD, whilst modifiable risk factors are poor socioeconomic status, high blood pressure, high body mass index (BMI) ,elevated blood lipid levels, hyperglycaemia , physical inactivity, smoking and poor diet (6-9). Among the modifiable risk factors, diet is one of the main contributors to the development of CVD and, therefore, improving dietary behaviour can be a strong preventative strategy against the disease (10). Current dietary recommendations in the UK provided by Scientific Advisory Committee on Nutrition(SACN) (11) states based on latest scientific evidence that, diet rich in fruits, vegetables, wholegrains and oily fish and low in saturated fat, trans-fat, carbohydrates and salt can help to maintain cardiovascular health (12-18). In 2016, SACN also concluded that increased intake of fibre, specifically wholegrain and cereal fibre was strongly associated with lower risk of cardio-metabolic disease(11). Additionally, higher intake of oat bran and β-glucans were reported to lower blood pressure, total cholesterol (TC), LDL–cholesterol (LDL-C) and triacylglycerol concentrations (TAG) (11). As a result, SACN increased the recommended intake values for fibre from 23g to 30g and stated that mean intakes of the population are well below the recommendation (14 g/day in adults aged 19–64 years, 13.4 g/day in adults aged 65+ years)(19). The European Food Safety Authority (EFSA) also approved the health claim, which states that the consumption of β-glucan at least 3g/d can reduce the blood cholesterol concentrations of healthy, and mildly hypercholesteraemic adults (20). In addition to fibre, wholegrains provide a range of nutrients including vitamins, minerals and bioactive polyphenols (21). Observational and human intervention studies have shown evidence for cardiovascular health benefits of consumption of fruits, vegetables and wholegrains, which may be due in part to their high content of polyphenols (22). Although the fate of these compounds after ingestion and their mechanisms of action are not completely understood,

there is a growing body of evidence suggesting their protective role against cardiovascular disease risk factors (22-24). Oats are extensively studied for their cardiovascular health benefits mostly due to their high β-glucan content, but they also contain substantial amounts of phenolic acids, which are a sub-class of polyphenols, including the most abundant ferulic acid and structurally similar avenanthramides (25, 26). There are limited number of in vitro and human studies showing cardio protective effects of these compounds either in oats or in other plant sources(27-31). However, further human studies are necessary to explore the role of oat phenolics (phenolic acids and avenanthramides) on cardiovascular health.

This literature review will mainly focus on the aetiology of CVD and its risk factors, how endothelium maintains vascular health, impact of wholegrain consumption on CVD risk factors and the potential of plant polyphenols in particularly phenolic acids in the prevention or management of the disease.

1.2 Cardiovascular Disease

CVD represents a group of pathophysiological conditions of heart and/or blood vessels such as coronary heart disease, stroke, hypertension, congestive heart failure and myocardial infraction, all majorly caused by atherosclerosis(32). Atherosclerosis develops slowly over many years and its consequences are mostly preventable with early detection and appropriate lifestyle changes(1, 32). A normal, healthy vascular system is fundamental to maintain the cardiovascular health and it is necessary to understand the pathophysiology of CVD including atherosclerosis and endothelial dysfunction to develop preventative strategies(33).

1.2.1 Underlying Pathophysiology: Endothelial Dysfunction and Atherosclerosis

Endothelium is a single layer of cells that lines the intima of artery walls and plays a vital role in the regulation of vascular homeostasis(34, 35). In a healthy endothelium, relaxed vascular tone and low levels of oxidative stress are mainly maintained by the release and balance of mediators including nitric oxide (NO), prostacyclin (PGI2) and endothelin (ET-1) and also with controlled local angiotensin-II activity (35). NO, which is synthesised by endothelial NO synthase (eNOS), is an important signalling molecule responsible for vasodilation and has important effects on the vessel wall such as promoting the relaxation of the arteries and preventing the proliferation of smooth muscle cells, inhibiting the activation and aggregation of platelets and the inflammatory leukocyte adhesion (36, 37). PGI2, another signalling molecule acts synergistically with NO to inhibit platelet activation (**Figure 1.1 A**) (36). On the other hand, vasoconstrictors ET-1 and angiotensin II are responsible for smooth muscle cell proliferation and increase oxidative stress(35, 36). When the endothelial cell layer is injured or there is an imbalance between the mediators of vasodilation and vasoconstriction such as reduced bioavailability of major vasodilator NO, endothelial dysfunction occurs and it is considered as an early

sign of atherosclerosis (36, 38). Endothelial dysfunction is associated with increased expression of vascular cell adhesion molecules (VCAM), increased oxidative stress, and increased synthesis of pro-inflammatory and pro-thrombotic factors, platelet activation, smooth muscle cell activation and modulation of vascular tone(**Figure 1.1 B**)(36).

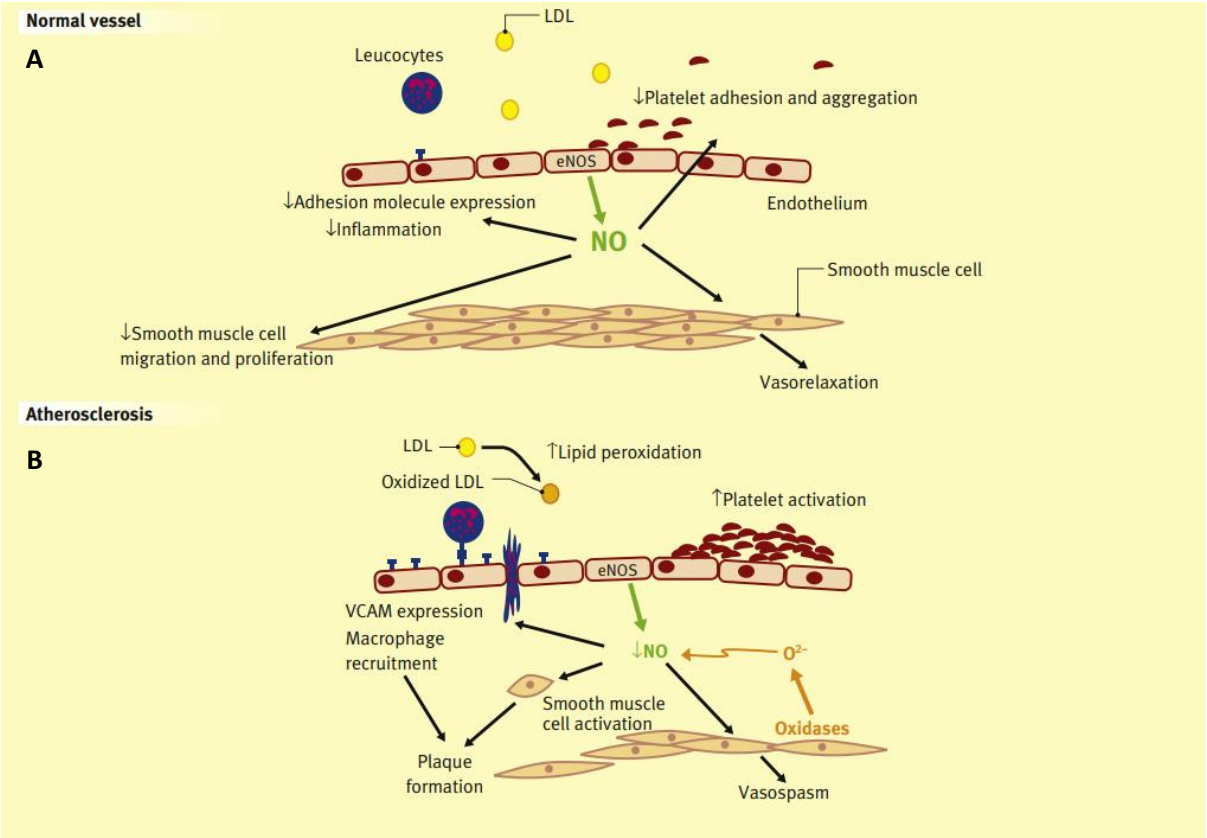


Figure 1.1 Endothelial function and dysfunction

A-endothelial function in healthy vessels. **B**-endothelial dysfunction in atherosclerosis. (Adapted from Douglas et al, 2010)(36)

The development and progression of atherosclerosis can be considered as a form of chronic inflammation(39). The condition usually starts with an increase in endothelial permeability to lipoproteins and adhesiveness of leukocytes and platelets causing atherosclerotic lesions and as described above, endothelial dysfunction (**Figure 1.1 B** and **Figure 1.2 A**) (40). Foam cells are macrophages that form the fatty streaks together with lipid-laden monocytes and T-lymphocytes (41). These inflammatory cells are joined by the proliferation and migration of smooth muscle cells and forms the intermediate lesions (**Figure 1.2 B**) (40, 41). The formation of advanced lesions of atherosclerosis result in fatty streaks (39). Fibrous cap forms and causes the further enlargement and restructuring of the lesion (40). Accumulation of inflammation to the area increase the number of macrophages and lymphocytes and leads to the release of cytokines, chemokines, growth factors and

hydrolytic enzymes (40, 42, 43). These cells may then lead to the formation of a necrotic core underneath the fibrous cap, which results in necrosis, apoptosis, lipid accumulation and proteolytic activity (**Figure 1.2 C**) (39, 44). The rupture of fibrous cap, degradation of the whole matrix by proteolytic enzymes and haemorrhage from the lumen followed by thrombosis can result in the occlusion of the artery (**Figure 1.2 D**) (40).

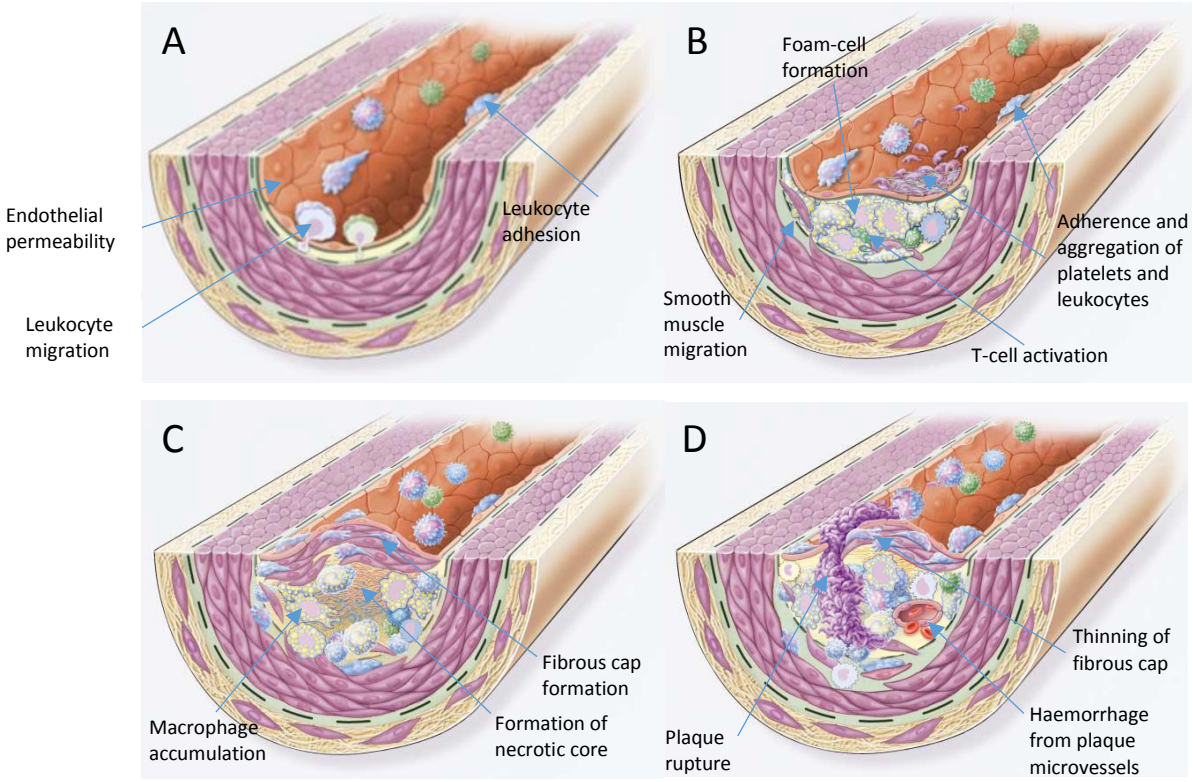


Figure 1.2 Progression of atherosclerosis (Adapted from Ross et al., 1999)(40).

1.2.2 Cardiovascular Disease Risk Factors

1.2.2.1 Plasma lipids and blood pressure

A normal plasma lipid profile and lower blood pressure are important factors involved in cardiovascular health (45). Cholesterol is transported in plasma lipoprotein particles via circulation and it is required for various biological processes such as making bile acids, hormones and providing cell membrane support (46). An atherogenic lipid profile can be characterized by high levels of TC, LDL-C, TAG (47) and low levels of high density lipoprotein cholesterol (HDL-C) (45, 48, 49). Statin therapy has been shown to effectively reduce the TC and LDL-C levels mainly by inhibiting 3-hydroxy-3-methylglutaryl-

coenzyme A reductase , a regulatory enzyme in cholesterol biosynthesis(50). It has been estimated that a 10% reduction in plasma cholesterol leads to a decrease in coronary artery disease risk of 50% at age 40, 40% at age 50, 30% at age 60, and 20% at age 70 (45). National Health Service (NHS) in the UK recommends the following target levels of cholesterol to people at high CVD risk: TC< 4mmol/l, LDL-C< 2 mmol/l, TAG<1.7 mmol/l and HDL-C>1 mmol/l (51). Research suggests that a healthy diet has a significant role in the maintenance of normal lipid profile (52). A 1% reduction of energy from saturated fat or trans-fat and replacement with n-6 polyunsaturated fat can lead to a decrease in LDL-C (- 0.05 mmol/L) (53, 54). Additionally, replacement of same amount of saturated fat with unrefined carbohydrates may also be beneficial but further research is necessary as the findings are conflicting (53, 54). Substitution of low glycaemic index carbohydrates with high glycaemic index carbohydrates has been reported to decrease TAG levels by 12-25% (52, 55, 56).On the other hand , consumption of fish oil (omega-3 poly-unsaturated fatty acids , 3.5 g/day) is effective in lowering TAG levels (-0.45 mmol/ L)(57). Soluble fibre (3.5 g/day) such as oat B-glucan can also lower LDL-C concentration (- 0.19 mmol /L) (58). Plant sterols and stanols (2 g/day) have shown to lower LDL-C by about 10% (59). Finally, a recent meta-analysis of RCTs highlighted the association between flavanol-containing tea, cocoa, and apple products and lower levels of TC (-0.21 mmol/L), LDL-C(-0.23 mmol/L) and TAG(-0.11 mmol/L) and higher levels of HDL-C (0.15 mmol/L)(60).

Blood pressure (BP) is another strong risk factor for CVD, with higher BP being linked to higher incidence of CVD (61). Based on recent revisions, BP can be categorized as follows: Normal BP (Systolic BP (SBP) <120 mm Hg and Diastolic BP(DBP) 80 mm Hg), elevated BP (SBP 120-129 mm Hg and DBP<80 mm Hg), stage 1 hypertension (SBP 130-139 mm Hg or DBP 80-89 mmHg) and stage 2 hypertension (SBP ≥140 mm Hg or DBP ≥90 mm Hg)(62). Evidence is suggesting a higher risk of CVD for SBP levels from <115 mm Hg to 180 mmHg and DBP levels from 75 mm Hg to 105 mm Hg(63). However, a 2 mm Hg reduction in SBP may result in a 10% decrease in stroke mortality and 7% decrease in heart disease and other vascular causes mortality (63). There are numerous classes of antihypertensive agents, which can be used in the treatment of hypertension. These agents include thiazide diuretics, angiotensin converting enzyme inhibitors, angiotensin II receptor blockers and calcium channel blockers(64). On the other hand, a solid body of evidence states that healthy lifestyle changes including diet can prevent hypertension in normotensive subjects and help to reduce BP in hypertensive subjects(64). There is a well-established relationship between salt reduction and BP, a 5g of reduction dietary sodium intake can lead to 1-2 mmHg lower SBP in normotensive subjects and 4-5 mmHg lower SBP in hypertensive subjects (65, 66). Heavy alcohol consumption is another contributor of sustained elevated levels of BP and limitation of 20-30g of ethanol per day in men and 10-20g of ethanol per day in women is suggested to be necessary (64, 67, 68). Moreover, a diet rich in fruits , vegetables, soluble fibre, wholegrains, low fat dairy products and plant proteins is important for the reduction of BP (69).

Total polyphenol intake assessed via the amounts excreted in urine was also negatively associated with hypertension (70).

Although there are effective therapeutic treatments available to control CVD risk factors, it is not manageable in terms of costs and potential health complications to treat all subjects with drugs. Therefore, preventative impact of lifestyle interventions such as regular physical activity, non-smoking, maintaining a healthy BMI and healthy diet high in fruits and vegetables are key to manage these risk factors (33).

1.2.2.2 Nitric Oxide bioavailability and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase activity

As briefly described above, NO is an important signalling molecule that has various roles in physiological and pathological processes including the endothelium (71). NO is synthesised from the amino acid L-arginine through the L-arginine nitric oxide synthase (NOS) pathway by eNOS (72-74). An alternative way of generating this molecule *in vivo* has more recently been suggested as the nitrate-nitrite-nitric oxide pathway which consists of oxygen-independent and NO-synthase-independent single electron transfer reactions (75-77). The vascular effects of NO include the maintenance of vascular homeostasis. This can be described by a continuous and basal release of the molecule at low levels in the endothelium via cGMP-mediated relaxation of vascular smooth muscle cells, resulting in vasorelaxation (73, 78). Therefore, NO is an important regulator of blood pressure and blood flow (79).

NADPH oxidase is a membrane bound enzyme complex consisting of a guanosine triophosphate (GTPase) subunit and different phox subunits. NADPH oxidase can be expressed in various ways in the cardiovascular system and subunits of the enzyme can be activated in the events of cardiovascular diseases (80). Although moderate activity of NADPH oxidase is important for angiogenesis and immunity, excessive reactive oxygen species (ROS) produced by an overactive NADPH oxidase may lead to endothelial dysfunction (81-83). The primary function of NADPH is to generate superoxide and/or hydrogen peroxide (H_2O_2). Reduction of O_2 to generate a superoxide anion ($O_2^{\bullet-}$), is considered to be the major source of ROS and this reaction is catalysed by NADPH oxidase. The secondary ROS is then produced with the direct or enzyme catalysed interaction of $O_2^{\bullet-}$ and other molecules. Reduction of $O_2^{\bullet-}$ leads to the formation of H_2O_2, which is further converted into hydroxyl $HO^{\bullet}$. $O_2^{\bullet-}$ can also react with NO to form peroxinitrite ($ONOO^-$), ultimately leading to the uncoupling of eNOS and subsequently, a reduction in the production of NO, generation of superoxide and an increase in oxidative stress (84). **Figure 1.3** illustrates the role played by NADPH oxidase activity and NO bioavailability in increasing oxidative stress, a known factor in vascular disease.

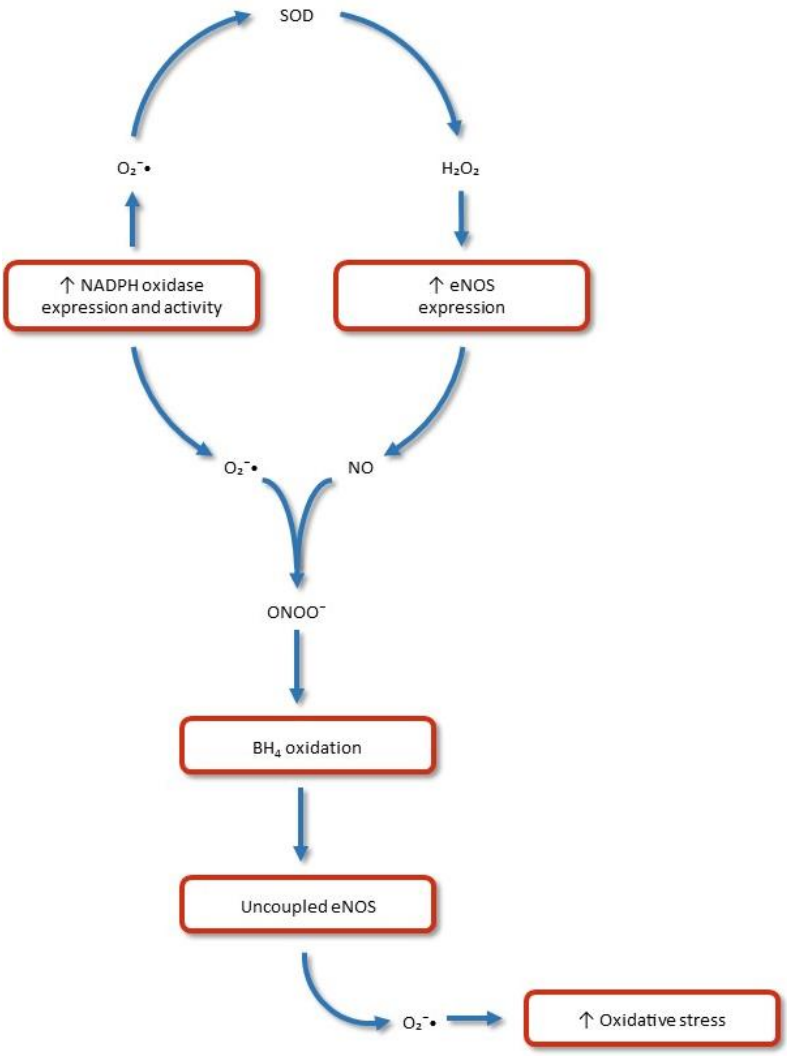


Figure 1.3 The role of NADPH oxidase activity and NO bioavailability in increasing oxidative stress

NADPH oxidase, the main generator of superoxide ($O_2-\bullet$), and eNOS expression are major factors upregulated in the pathophysiology of vascular disease. Hydrogen peroxide (H_2O_2) is produced with the dismutation of $O_2-\bullet$, mediated by superoxide dismutase (SOD) and can lead to increased eNOS expression. $O_2-\bullet$ generated through NADPH oxidase activity and NO produced through increased eNOS activity react to form peroxynitrite ($ONOO-$). $ONOO-$ oxidises tetrahydrobiopterin (BH_4), an essential cofactor of eNOS, causing uncoupling of eNOS. As a result, functional NO-producing eNOS is converted to a dysfunctional $O_2-\bullet$-generating enzyme which increases oxidative stress in the vascular system. Figure adapted from Förstermann and Sessa (85).

1.2.2.3 Extracellular Vesicles (EVs)

EVs are membrane-bound vesicles released from cells as result of cellular stress, cell activation and apoptosis and their roles include intercellular communication between cells with the of transport cargo that contains DNA, RNA and proteins(86, 87). EVs are mainly classified into different classes depending on their biological function, cellular origin or their biogenesis(88). The three main classes of EVs are exosomes, microvesicles and apoptotic bodies(88). Exosomes are considered to be the smallest vesicles (~50-150nm) and are produced in structures called multivesicular bodies which facilitate their transfer into extracellular environment by exocytosis through the fusion with plasma membrane(89). Microvesicles (~100nm-1 μm) that contain cytoplasmic cargo, bud from plasma membrane and cause cellular activation or stress (86, 89). While some of the microvesicles can be characterized with the expression of phosphatidylserine serine (PS) on their outer membrane, they can also be identified with the expression of a surface antigen arising from their parent cell (90). For instance, microvesicles derived from platelets express CD41 antigen whereas endothelial derived microvesicles can be characterized by the expression of CD105, CD31 or CD62e (91, 92). There are also apoptotic bodies (~200 nm–5 μm) which are being generated from apoptosing cells and overlap in size with microvesicles (89).

EVs are considered as potent mediators of both physiological and pathological processes (93). Depending on their numbers in the circulation, composition and content which represent particular signs of cell activation and injury, they can be used as potential biomarker of diseases including CVDs (87, 94). In the process of endothelial dysfunction, endothelial injury has shown to trigger the release of EVs (95, 96). Accordingly, subjects with CVDs show significantly high levels of circulating endothelial cell derived EVs (97). However, it is not only the release of EVs that reflects vascular dysfunction, when a vascular cell is targeted by EVs and incorporated via ligand or receptor signalling, through its plasma membrane or fusion of vesicle, EV cargo that contains cytokines, mRNA, noncoding RNAs and transfer proteins is released into target cell nucleus or cytoplasm (87, 95, 98). Thus, the phenotype and biological function of recipient vascular cell is influenced and this can contribute to CVD progression (95). For example, circulating endothelial microvesicles were reported to carry eNOS which originated from their parent cell and have crucial role in NO bioavailability (99). Elevated levels of circulating CD31+ /AnnexinV+(PS+) EVs were reported in CAD patients and circulating levels of CD62e+ endothelial EVs were shown in patients with pulmonary hypertension(100, 101). Increased numbers of EVs have been associated with CVDs such as coronary artery disease (CAD) peripheral artery disease and CVD risk factors such as hypertension (97, 102, 103). Platelet derived EVs (PDEVs) which form the majority of EV population in plasma with procoagulant activities, were associated with atherosclerosis and CAD and linked to CVD risk factors such as hypertension and abnormal lipid profile(103-107). On

the other hand, elevated levels of endothelial derived EVs (EDEVs) which mainly originates from activated and apoptotic endothelial cells were correlated with endothelial dysfunction in CAD patients(108). Increased numbers of EDEVs were also shown in patients with hypertension, heart failure, dyslipidaemia and atherosclerosis (109-111). These promising evidence support the notion that EVs have the potential to contribute to the progression of CVDs and they can be considered as emerging CVD risk factors.

1.3 Dietary Polyphenols

1.3.1 Classification, Chemical Structure and Dietary Sources of Polyphenols

There is a growing interest in the role of polyphenols including flavonoids and phenolic acids as protective agents of the human diet. Polyphenols are present as secondary metabolites in plants and have various roles including protection against herbivores, microbial infection, and UV light, attraction of pollinators, seed-dispersing animals and cell signalling to regulate nitrogen-fixation (112). Flavonoids contain 15 carbons with two aromatic rings connected by a three-carbon bridge and consist of C_6–C_3–C_6 structure with the main subclasses including flavones (parsley and celery) , flavonols (tea, apples and onions) , flavan-3-ols (tea, cocoa, red grapes and red wine), isoflavones (soybeans and legumes), flavanones (citrus fruits), and anthocyanidins (berries and red wine) (**Figure 1.4**). Phenolic acids are also present in some plants as non-flavanoid polyphenolic compounds. Main phenolic acids are hydroxybenzoic and hydroxycinnamic acid derivatives with C_1-C_6 and C_3-C_6 structure, respectively(113). Coffee contain high levels of hydroxycinnamates, particularly chlorogenic acids such as 5-caffeoylquinic acid(114). After coffee, wholegrains such as oats are the second richest dietary source of phenolic acids , containing in particular the hydoxycinnamate ferulic acid (115). The mean dietary intakes of flavonoids is 352mg/d in the UK, flavanones being 9 mg/d, flavan-3-ols 110mg/d, flavanols 28mg/d, flavones 2mg/d , isoflavones 1mg/d, anthocyanins 100mg/d and phenolic acids 669mg/d(116-118).

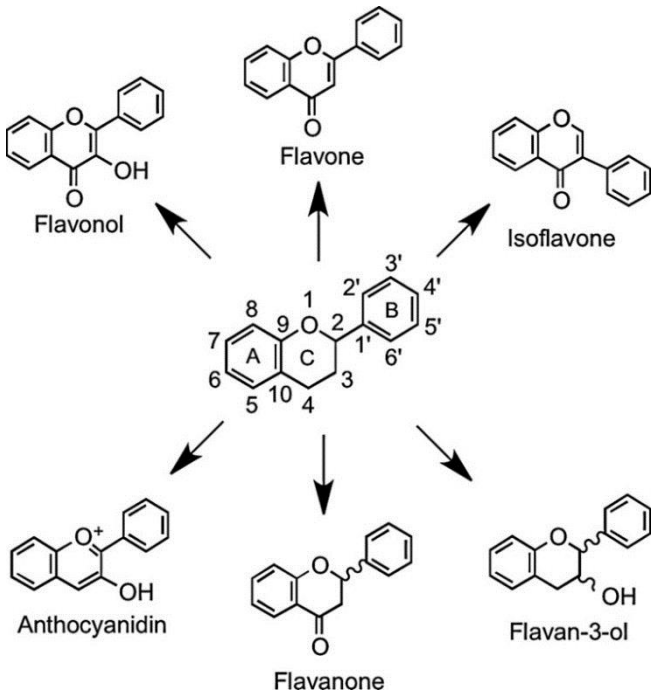
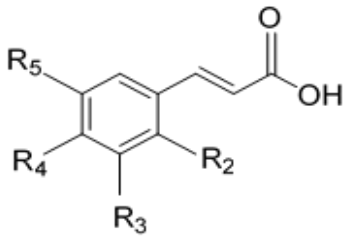


Figure 1.4 Structure of the main flavonoid skeleton and subclasses (23).

1.3.2 Phenolic Acids and Avenanthramides in Oats

Phenolic acids and avenanthramides contain one aromatic ring bearing an acid group and one or more hydroxyl groups. Phenolic acids 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 (119, 120). Hydroxybenzoic acids and hydroxycinnamic acids are the two classes of phenolic acids found in oats (**Figure 1.5**). Hydroxybenzoic acid derivatives present in oats include, protocatechuic, syringic, vanillic , *p*-hydroxybenzoic and gallic acids, while hydroxycinnamic acid derivatives are ferulic, p-coumaric, o-coumaric, caffeic and sinapic acids. (121, 122) 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, avenanthramide -B and avenanthramide -C, which occur in the bran or outer layers of kernel (123). Typical amounts of hydroxycinnamic acids, particularly, ferulic and p-coumaric acids are ranging from 96.2-327 µg/g and 56.7-109 µg/g, respectively in oats. For hydroxybenzoic acids, namely vanillic and syringic acids, the amounts are ranging between 25.7-119.7 µg/g and 31-59.9 µg/g respectively. For avenantramides, the reported quantities are between 42-91 µg/g.(115)



Cinnamic acid derivatives

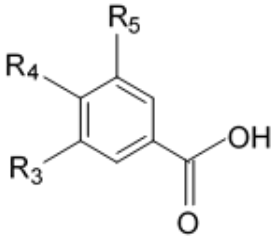
R_2=OH; o-coumaric acid

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

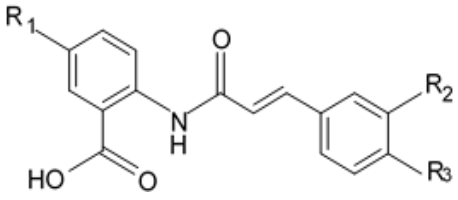
R_3=R_4=R_5=OH; gallic acid

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 1.5 Structures of phenolic acids (cinnamic and benzoic acid derivatives) and avenanthramides.

1.3.3 Absorption and Metabolism of Polyphenols: A Focus on Wholegrain Phenolic Acids and Oat Avenanthramides.

Before evaluating the bioactivity of polyphenols in the body, one needs to consider the absorption and metabolism in order to understand the fate of these compounds before reaching to the blood stream to exert potential health benefits. After ingestion, the modification of the polyphenol glycosides starts in the oral cavity by the enzymes in saliva to a limited extend and then most of them reach to small intestine after passing through the stomach and thereafter the colon (22) (**Figure 1.6**). Many enzymes play role in the hydrolysis and absorption of these compounds within the gastrointestinal tract. Most importantly, lactase phloridzin hydrolase (LPH) in the brush border of epithelial cells of the small intestine cleaves the glycoside unit of the molecules. LPH has a broad specificity for flavonoid-O-B-D-glucosides and the released aglycones are highly lipophilic which helps them to enter the epithelial cells via diffusion (124). Cytosolic B-glucosidase is another enzyme that mediates glycoside hydrolysis with the involvement of the active sodium-dependent glucose transporter (SGLT-1), which might be involved in the transportation of flavonoid glycosides into epithelial cells(125).

After absorption, polyphenols are subjected to phase II enzymatic metabolism where they can be conjugated with sulphate, glucuronic acid and methyl groups with the catalysis of specific enzymes such as UDP-glucuronosyltransferases , sulphotransferases and catechol-O-methyltransferases(23, 126). Following the phase II metabolism that initially takes place in the wall of the small intestine, metabolites are passing to the liver via portal vein where they might be subject to further phase II metabolism before undergoing renal excretion (127). It has been estimated that only 5-10% of total polyphenol intake is absorbed in the small intestine, whilst the remainder may persist to the colon, where it is metabolised by the gut microbial community (128, 129) Decarboxylation, reduction, demethylation and dihydroxylation reactions are common bacterial reactions which form low molecular weight metabolites from original structures that can be absorbed, undergo further phase II metabolism locally and/or in the liver before entering the systematic circulation where the tissue exposure and uptake can take place for the exertion of some of the health benefits (130, 131). Additionally, some of the carbon glycosides and flavonoid A ring might be converted to short chain fatty acids as a source for energy metabolism by the host and exhaled as CO_2 (132). Factors such as age, sex, genetic polymorphisms, pathophysiological status, individual variability of composition of gut microbiota, food matrix and food processing may affect the bioavailability of polyphenols and introduce inter-individual variability in response to interventions (23, 133).

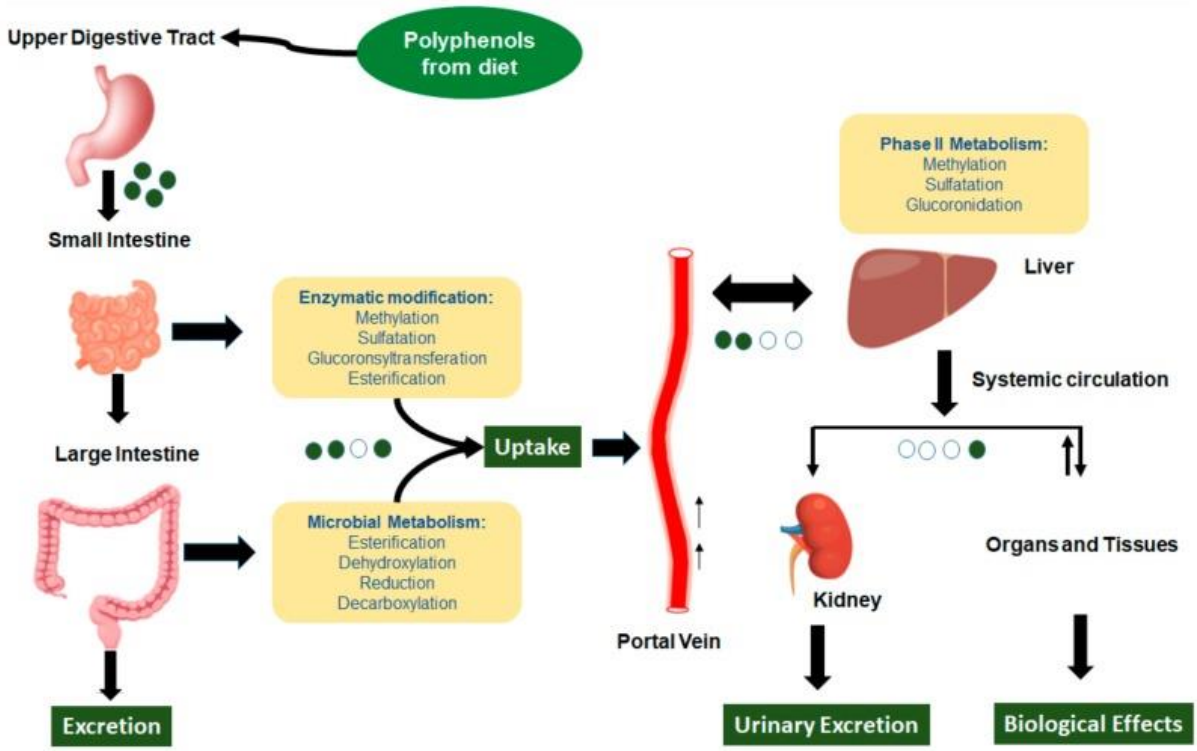


Figure 1.6 Schematic representation of the absorption and metabolism of polyphenols. Green dots are aglycones and white dots are metabolites. Figure adapted from Cipolletti et al, 2018(134).

To focus on hydroxycinnamates that are present in wholegrains, both *in situ* and *ex vivo* experiments show that p-coumaric acid, ferulic acid and caffeic acid can be absorbed in the stomach, jejenum, ileum and colon of rats (135-139). Fast gastric absorption of p-coumaric acid and ferulic acid in rats and only free ferulic acid in humans have been reported in the gastrointestinal tract (136, 140). The type of hydroxycinnamate highly affects the absorption rate with ferulic and p-coumaric acids having higher absorption efficiency than caffeic acid in caco-2 cell lines (141-144). Although the exact mechanism of absorption is not well understood, facilitated diffusion suggested to play a role (141). To detail the metabolism of ferulic acid, it is mainly absorbed in the foregut and sulphated and or glucuronidated in the liver (135, 145). Some studies have shown that intestinal mucosa and kidneys might also play a role in the conjugation process (138). Intake of high doses of the compound might saturate this pathway and also increase its secretion via bile which can then reach the colon and be metabolized by gut microbiota(146). Ferulic acid can be converted into m-hydroxy-phenylpropionic acid with demethylation, reduction and dehydroxylation in the gut(147). Furthermore, it can be metabolized via B-oxidation in the liver and be accounted for urinary metabolites such as vanillic acid and dihydroferulic acid in rats (146-148).As stated previously, hydroxycinnamates also found in the bound form in oats as esterified with hydroxy acids, sugars and polymers. The metabolism of bound hydroxycinnamates are different than free forms, esterases located in the lumen of the intestine can hydrolyze the bound hydroxycinnamates or fibre-phenolic bond can be cleaved by intestinal microbiota to generate free forms which can then be absorbed and further metabolized (141, 149).

Human studies that examined the absorption, metabolism and bioavailability of phenolic acids in wholegrains reported the concentration in the blood and urine at the peak times (150, 151). Kern et al., investigated the absorption of hydroxycinnamic acids from high-bran wheat based breakfast cereal in 6 subjects (40 g portion containing 130 mg of phenolics). The results of this study showed an increase in plasma ferulic and sinapic acid concentrations (200 and 40 nM, respectively) with absorption peaks between 1 and 3 h suggesting that absorption might primarily take place in the small intestine from the soluble phenolics. These findings suggested that covalently bound diferulic acids are only absorbed in small amounts (<10nM in plasma), showing that the bulk of ester-linked compounds can be further metabolized by bacteria in the colon or excreted in the feces. Additionally, increases of 4 fold in ferulic acid and 5 fold in feruloylglycine in 24h has indicated the urinary excretion of hydroxycinnamates and the form of ferulic acid present both in plasma and urine was found as conjugates(feruloylglycine and/or glucuronides) (151). On the other hand, Bresciani et al., looked at the absorption, metabolism and bioavailability of phenolic acids in wholegrain bread (WGB) (94 g portion containing 87 mg of ferulic acid) or bread enriched with an aleurone fraction, AB-94 and AB-190 (94 and 190g portions containing 43 and 87 mg of ferulic acids, respectively) in 15 subjects. The metabolites found in plasma and urine were in the form of sulfate and glucuronide conjugates of phenolics. Plasma kinetics showed a peak at 1 h for ferulic acid-4'-O-sulphate,76.6 nmol/L (AB-94) and a peak at 7 h for dihydroferulic acid 4'-O-sulfate ,11.9 nmol/L (AB-190). Additionally, urinary excretion of ferulic acid 4-O-sulphate between 0-3h indicated an early absorption in the small intestine, excretion of sinapic acid sulfate and ferulic acid 4-O-sulphate between 3-6h showing the release of the most accessible fibre-linked phenolic acids by microbiota leading the increased absorption and finally vanillic acid excreted between 6-48h indicated a deeper microbiota catabolism (150). A 3-way crossover avenanthramide bioavailability study carried on 6 subjects with the consumption of 360mL skim milk (control) , 0.5 or 1g avenanthramide enriched mixture showed that oat avenanthramides are also bioavailable. Maximum plasma concentrations of avenanthramides after consumption of 0.5 and 1 g AEM were 112.9 and 374.6 nmol/L at around 2h after consumption. However it must be noted that the amounts provided in this study was really high and cannot be consumed in a portion of wholegrain oats (27). Finally, a pilot study that we previously performed in our group (see Appendix), reported that intake of oat bran (60g, 2.5 mg of avenanthramides and 28.6 mg of phenolic acids) resulted in elevated levels of urinary excretion of 30 phenolic acid metabolites, mainly between 0-2 and 4-8h in 7 healthy men. The major excreted metabolites were vanillic acid, 4- and 3-hydroxyhippuric acids and sulphate conjugates of ferulic and benzoic acids. The findings showed that free and conjugated phenolics can be absorbed early (0-2h) from the small intestine and bound phenolics are subjected to the quick actions of gut microbiota (4-8h)(152).

1.4 Dietary Polyphenols and Vascular Health

1.4.1 *In vitro* bioactivity of polyphenols and their metabolites in relation to cardiovascular diseases.

Representative studies investigated the mechanisms of action exerted by polyphenols or their metabolites in cell culture models in relation to cardiovascular diseases are summarized in **Table 1.1**. There are several proposed mechanisms in relation to interactions of flavonoids with NO, key regulator of vascular health. The NO related non-specific mechanisms were described by high antioxidant nature of phenolic groups and the reaction of these groups with superoxide and other reactive oxygen species which can reduce NO breakdown (153-155). However, there is insufficient evidence to support this mechanism of action due to the lack of data emerging from studies that have utilised physiological metabolites of polyphenols at adequate concentrations *in vivo* (126). On the other hand, there is an accumulating evidence showing the inhibitory or stimulatory actions of polyphenols or their metabolites in protein kinase and lipid kinase signalling pathways such as phosphoinositide 3-kinase , Akt/protein kinase B and mitogen activated protein kinase which might be resulting in increased eNOS activity and expression and hence increased NO production (81, 156). Finally, polyphenols, or their circulating phenolic metabolites, might also have an inhibitory action on enzymes involved in the generation of reactive oxygen species (ROS), such as NADPH oxidase, xanthine oxidase and lipoxygenase, and this can prevent NO breakdown (153, 157). Specific mechanisms related to NO can be more dependent on particular flavonoid structure and some of the proposed mechanisms include increased eNOS activity and expression and hence increased NO production (81).

Huang et al. showed that the 50 μM of dihydrocaffeic acid increased scavenging of ROS ability and activity of NO synthase in human-derived EA.hy926 endothelial cells but the concentration tested was higher than the values observed in plasma after ingestion (158). A recent study by Claude et al. addressed this issue and showed the inhibitory effects of 4'-O-methyl(-)-epicatechin (at 0.2,0.5 and 2 μM with a magnitude from -21 to-33%), 4'-O-methyl(-)-epicatechin-7-β-d-glucuronide (at 2 μM by about -14%), and (-)-epicatechin-4'-sulfate (at 0.2 and 0.5 μM from -18% to -27%) on human aortic endothelial cells (159). The findings from this study has shown that these flavanol metabolites can help to preserve vascular endothelium integrity with decreasing the adhesion of monocytes to endothelial cells (159).On the other hand, 10-40 μM protocatechuic acid, a colonic catabolite of cyanidin-O-glucoside, has been shown to inhibit monocyte adhesion to tumour necrosis factor-alpha activated mouse aortic endothelial cells (MAECs) and to reduce vascular and intracellular adhesion molecule (VCAM1 and ICAM1) levels both in MAECs and in Apo-E deficient mice, a well-established animal model of atherosclerosis (160). For example, one of the most potent flavanols, quercetin and its metabolites, methyl-quercetin and quercetin glucuronide have been tested *in vitro* (5 and 10 μM) on human

endothelial aortic cells and the results suggested an increase in the phosphorylation of eNOS via AMPK pathway and the production of NO (161).The inhibitory effects of (-) epicatechin and its metabolites were tested in vitro on NADPH oxidase activity with using human umbilical vein endothelial cells (HUVEC) and cell lysates. The findings suggested that (-) epicatechin is an $O_2^{\bullet -}$ scavenger but not an inhibitor of NADPH oxidase whereas the opposite results was obtained for the 3'- and 4'-*O*-methyl epicatechin. The dimer procyanidin B2 and (-) epicatechin were also shown to be $O_2^{\bullet -}$ scavengers but also the inhbitors of the enzyme (28).

The protective effects of polyphenolic compounds on CVDs is promising but there are important factors need to be considered when establishing these effects in *in vitro* models. Polyphenols are metabolized in the human body after ingestion and they mostly appear in the system as methyl, sulfate and glucuronide metabolites which can change their fate. Moreover, their concentration in the plasma rarely exceeds nM/L after normal dietary intake (162). Additionally, these compounds might not be absorbed in the small intestine but degraded to smaller phenolics with the action of bacteria in the colon and then absorbed into the blood before being excreted (22). Biologically relevant concentrations and the form of the compounds that are likely to occur in the vascular system should be taken into account when performing *in vitro* experiments on different cell lines. It is also important to use human metabolites with human cell lines and animal metabolites for animal cells to increase the accuracy of the results (22).

Table 1.1 Effects of polyphenols or their metabolites in cell culture models in relation to cardiovascular diseases.

Signaling/effect activation;⬆, signaling/effect inhibition;⬇ , - expression inhibition ↔ , HUVECs, human umbilical vein endothelial cells; SMC, smooth muscle cells, ICAM-1,intercellular adhesion molecule 1; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule 1.

Class	Metabolite	Outcomes	Model	Refs
Anthocyanins	Protocatechuic acid (3,4-dihydroxybenzoic acid)	Monocyte adhesion ⬇	Mouse endothelial cells	(160)
		VCAM1 &ICAM1 ↔	Apo-E mice	(160)
		Shear-stress related platelet aggregation ⬇	Platelets	(163)
Hydroxycinnamates & Avenanthramides	Dihydroferulic acid 3-(3'-hydroxyphenyl)propionic acid, chlorogenic acids	Hyperactivity of platelets ⬇	Human Platelets	(119, 158, 164, 165)
	Dihydrocaffeic acid	Scavenge intracellular ROS NO synthase ⬆	Human-derived EA.hy926 endothelial cells	
	Avenanthramides A, B and C	Monocyte adhesion ⬇ ICAM1 - , E-selectin- , VCAM1 ↔ Cytokines ⬇, SMC proliferation ↔ NO ⬆, eNOS ⬆	Human aortic endothelial cells & smooth muscle cells	
Flavan-3-ols	4'-*O*-methyl-(-)-epicatechin, 4'-*O*-methyl-(-)-epicatechin-7-O-glucuronide, (-)-epicatechin-4'-sulphate	Monocyte adhesion⬇	Human aortic endothelial cells	(159)
Flavonols	3'- and 4'-O-methyl-quercetin	ICAM1 - , E-selectin- , VCAM1 ↔	Human aortic endothelial cells	(166)
	Quercetin-3'-O-sulfate, quercetin-3-O-glucuronide, isorhamnetin 3-O-glucuronide	Vasorelaxation NO↔ , NOS ↔	Rat thoracic aortic rings	(167)
Lignans, isoflavones	Equol	Vasorelaxation ⬆ NO ⬆ eNOS ⬆	Human aortic rings, HUVECs	(168), (169)

1.4.2 Epidemiological evidence of human studies

The evidence from epidemiological studies suggest that daily consumption of polyphenols may protect from CVDs (22). A meta-analyses of 14 prospective cohort studies has shown that consumption of anthocyanins, flavonols, proanthocyanidins, flavones, flavan-3-ols and flavanones is inversely linked to a reduction in the CVD risk of 11, 11, 10, 12, 13 and 12% respectively, when comparing quintiles of highest versus lowest intake (170). Another study reported the association between decreased CVD incidence/mortality and consumption of flavonoids in six out of twelve cohorts, flavonol consumption being the strongest association with CHD mortality (171). Cassidy et al., reported that high intake of anthocyanin is associated with a 32% reduction in the risk of myocardial infraction(MI) in 100,000 women, aged 25-45 years and followed up over 18 years(172). Additionally, Zamora-Ros et al has also shown that the link between high flavonoid consumption and 29% reduction in CVD mortality and in particular flavanones and flavanols reduced CVD mortality by 40 and 41%, respectively, in 40,000 subjects aged 29-69 years and followed up for 13 years (173). While promising evidence is accumulating on protective roles of polyphenols, it must be noted that some other epidemiological studies did not report a relationship between intake of polyphenols and CVD risk (174-176).

Limitations of these type of studies should be taken into account when considering the findings. There is a need for greater uniformity on a number of factors such as study design variability, potential cofounding factors by other nutrients and bioactives, dietary assessment tools used, flavonoid classes assed, population characteristics (age/sex/health status), length of follow up and also CVD endpoints chosen need to be considered carefully before definitive conclusions are made from epidemiological data (171).

1.4.3 Clinical Human Intervention Studies

The necessity of well powered and designed, double-blind randomized controlled trials (RCTs) is inevitable to confirm the observations made by epidemiological studies are causal. Blood pressure, endothelial function, vascular reactivity, arterial stiffness, and blood lipids are a range of prognostically validated surrogate markers of CVD risk commonly used in polyphenol intervention RCTs and will be discussed in this literature review.

1.4.3.1 Endothelial Function: FMD, NO and NADPH

The endothelium plays a key role in the regulation of vascular homeostasis and any functional and structural alterations correlate with CVD progression (177). Assessment of endothelial function can identify endothelial damage and provides prognostic data for risk degree in a later phase of the disease (34, 178). Endothelial function can be measured directly by assessing endothelial-dependent vasomotion, using flow-mediated dilation (FMD), or indirectly by circulating endothelial markers. FMD is widely considered as the 'gold standard' non-invasive technique to assess vascular function in cardiovascular research (179). It measures the ability of the arteries to dilate in response to endothelial NO release during reactive hyperemia induced by a 5-minute occlusion of the brachial artery (178). A meta-analysis of 23 RCTs reported that an improvement in FMD of 1% is associated with a decrease of 8-10% CVD risk over 4 years (180).

To date, a considerable amount of acute and chronic studies tested the protective effects of polyphenol-rich foods on endothelial function measured by FMD, with evidence being strongest for flavan-3-ols, cocoa and tea (23). A meta-analyses of 42 RCTs reported an improvement on FMD after both acute and chronic consumption of cocoa flavan-3-ols by 3.18% and 1.34% respectively (181). Another meta-analyses found that chronic tea consumption improved FMD by 2.6% when consuming a daily dose of 500 ml or 2-3 cups (182). The effects of berry polyphenols on endothelial function have been examined in a number of studies with reporting mixed results. A study conducted to test the vascular effects of cranberry juice consumption (835 mg total polyphenols and 94 mg anthocyanins per day) in patients with stable CAD, did not show an effect on FMD (183). Another study which looked at the acute consumption of a blueberry drink (766 mg of total polyphenols, 310–727 mg anthocyanin) by healthy young men reported improvements on FMD at 1,2 and 6 h post –consumption (184). An acute study which used flavanone intervention as both juice and whole orange (128-452 mg TPs, 107-352·80mg hesperidin), has shown the attenuation of vascular impairments measured as FMD in middle-aged healthy men(185). Daily consumption of hesperidin intervention (500 mg) for 3 weeks improved FMD by 2.5% in subjects with metabolic syndrome (186).

Emerging evidence from RCTs looking at the effects of polyphenols reported improvements on FMD, as discussed above. As FMD is a measure of in vivo endothelium derived NO bioavailability, it also shows the improvement in NO status (187). Indeed, a study of flavan-3-ol rich cocoa suggested to enhance vasodilation after consumption and this effect was shown to be reversed with a specific NOS inhibitor, administered intravenously (188). Elevated levels of NO have also been reported following both acute and chronic cocoa consumption (189, 190). Acute intake of pure (-) epicatechin and quercetin has also shown to improve the markers of NO and reduction in ET-1 in healthy men (191). In an RCT with healthy subjects, improvements in FMD were paralleled by the increase in NO levels following the intake of flavonoid-rich apples(192). Acute consumption of flavonoids and phenolic acids in champagne have also been shown to increase NO bioavailability and improve vascular function in 15 healthy adults (31). Finally, an acute study has reported that consumption of blueberry flavonoids (766, 1278, and 1791 mg doses) and their circulating small phenolic acid metabolites such as vanillic and homovanillic acids which have structural homologies to the pharmacologic NADPH oxidase inhibitor, apocynin were linked with decreases in neutrophil NADPH oxidase activity in healthy men (184).

There is a growing body of evidence suggesting the protective effects of polyphenols on endothelial function through increasing NO bioavailability. However, how exactly polyphenols exert these acute and chronic effects on NO status is still need to be elucidated. To understand the exact mechanisms of action, more cell and animal studies conducted with physiologically relevant doses and well-designed chronic human intervention studies are necessary.

1.4.3.2 Blood Pressure

Blood pressure is an important CVD risk factor, a lower blood pressure is linked to a healthier vascular system(193). It has been reported that a decrease in SBP of 2 mm Hg may lead to a 10% reduction in stroke mortality and 7% reduction in ischaemic heart disease and other vascular diseases(63). There are mixed findings on blood pressure and consumption of polyphenols. Similar to the evidence on endothelial function, the most promising results were reported for the consumption of cocoa and tea. A meta-analyses of 20 RCTs suggested that chronic intake of flavan-3-ol containing cocoa interventions lowered both SBP and DBP by 2 mmHg, mainly in healthy adults (194). Another meta-analyses comprising 11 chronic RCTs concluded that consumption of flavan-3-ol rich black tea can lower SBP and DBP by 1-2 mm Hg whereas green tea by 3 mm Hg (195). On the other hand, a meta-analyses of 11 RCTs reported that consumption of soy isoflavones were also shown to be effective in lowering both SBP (-2.5mm Hg) and DBP (-1.5 mm Hg) in hypertensive patients, but not in normotensive subjects (196). The evidence of anthocyanin rich foods such as berries on BP are inconsistent. While a meta-

analyses of 22 RCTs concludes a 2mm Hg reduction following the chronic intake of berries in SBP in subjects with CVD risk factors, a systematic review and few more RCTs reported no changes in overweight and hypertensive subjects, following the consumption of pure anthocyanin extracts or anthocyanin rich foods (197-200). Similarly, the evidence on grape polyphenols and red wine is inconsistent (22). For example, chronic intake of red wine by hypertensive subjects providing 280 and 560 mg of polyphenols did not change 24 h ambulatory blood pressure, a more reliable measurement of BP (201). Finally, non-flavanoid polyphenols of coffee mainly chlorogenic acids (400 mg) was inconsistently shown to have an effect on BP (202) and chronic intake of olive oil polyphenols such as hydroxytyrosol (30 mg/day) lowered BP in young pre-hypertensive women(203).

1.4.3.3 Microvascular Function, measured by Laser Doppler Iontophoresis

Microvascular dysfunction may be the cause of microvascular complications and vascular dysfunction that can subsequently precede to the development of atherosclerosis (204). Laser Doppler Iontophoresis (LDI) is a technique that measures the microvascular reactivity with focusing on the vasodilation of the forearm's peripheral microvasculature in response to a vasodilator called Acetylcholine (Ach) to measure endothelium dependent vasodilation and sodium nitroprusside (SNP) to measure endothelium independent vasodilation (205). A limited number of studies investigated the effects of polyphenols on microvascular function. Increased intake of flavonoid rich fruit and vegetables for 6 weeks were reported to improve endothelium dependent microvascular reactivity in men with CVD risk factors (206). Additionally, moderate consumption of champagne that contained flavonoids and phenolic acids (61.63 mg/l) suggested an acute improvement in endothelial independent microvascular function in healthy subjects (31). In contrast, acute consumption of blackcurrant juice (providing 50 mg of anthocyanins and 200 mg of phenolic acids) did not affect the microvascular function in healthy subjects (207). Daily consumption of green tea extract containing flavanols (714 mg/day, total polyphenols), for 3 weeks also did not change the microvascular reactivity in healthy men(208).

1.4.3.4 Arterial Stiffness

Arterial stiffness has been associated with the risk of CVD and often evaluated together with BP and endothelial function as all these parameters can have an effect on each other or modulated by oxidative stress and inflammation (209, 210). Pulse wave velocity (PWV) is considered to be the 'gold standard' technique to assess aorta stiffness whereas augmentation index (AIx) defined as the percentage of central pulse pressure related to reflected wave overlap in systole. AIx is also associated

with CVD risk factors and determined by age, SBP and heart rate (209). There are few studies examining the effect of different classes of polyphenols on arterial stiffness. For example, acute consumption of flavonoid-rich dark chocolate (2.62g of procyanidins per 100g) suggested to decrease Alx and improve FMD, without changing PWV in healthy subjects (211). Another study, examining the chronic effects of cocoa-flavanol containing drink (450 mg of flavanols) in both young and elderly healthy subjects, reported an improvement in FMD and PWV in both groups and a decrease in Alx only in elderly subjects (212). Conversely, chronic supplementation of pure (-)-epicatechin (100mg/d) and quercetin (160 mg/d) has failed to show an effect on both FMD and arterial stiffness in healthy subjects (213). One year supplementation with isoflavones (90mg /day) and flavan-3-ols (850 mg/day) has shown to improve PWV in patients with type-2 diabetes and post-menopausal women (214). Acute consumption of red wine polyphenols and chronic supplementation of cranberry juice (835 mg of total polyphenols and 94 mg of anthocyanins per day) have also been reported to decrease both PWV and Alx in CAD or CHD patients (183, 215). Finally, anthocyanin rich red orange juice (53.1 mg/L) was also reported to improve Alx acutely in healthy subject following the consumption of a fatty meal (216). However, there are numerous studies showing no effect on arterial stiffness after the consumption of cranberry juice, blueberry drink or pomegranate juice in healthy, obese or at CVD risk subjects(29, 217, 218).

1.4.3.5 Extracellular Vesicles

As previously described, EVs are released from cell types such as platelets and endothelial cells, as a result of cellular stress, activation and apoptosis and the elevated numbers are associated with pathologies related to CVDs including endothelial dysfunction(86, 87). There is a small body of evidence investigating the effect of polyphenols, majorly cocoa flavanols on the numbers of circulating EDEVs and PDEVs. A chronic study reported decreased levels of EDEVs following the consumption of high flavanol drink (375 mg) in CAD patients (99). Similarly, another chronic study testing natural cocoa bars (containing 701mg of flavanols) reported significant reduction of circulating EDEVs in women subjects (219). Consumption of cocoa flavanols (450 mg) for 2 weeks was also reported to decrease circulating EDEVs and correlated with decreases in SBP, Alx and inversely correlated with FMD improvements in both young and elderly subjects(220, 221). However, the same study failed to show any changes in the numbers of PDEVs following the consumption of cocoa flavanols (220). Another acute RCT providing relatively higher dose of flavanols (897 mg) has reported the decreased levels of PDEVs in healthy subjects(222). It is evident that flavanols may maintain endothelial integrity with improving FMD, other vascular measures and in parallel to these improvements, decreasing levels of circulating EDEVs and PDEVs. However, further *in vitro* studies examining the potential mechanism of action and more *in vivo* studies testing other polyphenol containing foods are necessary to understand the action of polyphenols on EVs.

1.5 Wholegrain Foods, Nutritional Composition, Types and Health Benefits

Wholegrain foods are defined by the FDA as consisting of the " intact, ground, cracked or flaked fruit of the grain whose principal components, the starchy endosperm, germ and bran, are present in the same relative proportions as they exist in the intact grain " (223). Wholegrains are a major source of dietary fibre that can be defined as carbohydrates that are resistant to digestion and absorption in the small intestine and partially or completely fermentable in the large intestine(224).Dietary fibre can be classified into two groups: soluble and insoluble, the former slows down the digestion process by dissolving in water and forming a gel that delays the gastric emptying whereas the latter does not dissolve in water and speeds up the passage of food and waste through the stomach (225).

Dietary phytochemicals defined as bioactives , non-nutrient plant compounds can also be found in wholegrains, including flavonoids, phenolics, carotenoids, lignans and vitamin E compounds (226, 227). Phenolics are the most complex and distinct type of phytochemicals in wholegrains including derivatives of cinnamic and benzoic acids and avenanthramides, present in oats (228). Ferulic acid and diferulates comprise by far the largest amount of phenolics in wholegrain foods and distinguishes them from other sources of phytochemicals (146, 229).

Although whole grains are a rich source of many nutrients, most cereals are less nutritious as they are being consumed after milling which is the removal of bran and germ and preservation of starch rich endosperm that is processed into flour (226, 230). In some countries, mandatory fortification might replace some of the lost minerals and vitamins from the grains (231). On the other hand, whole grains might be subjected to various types of processing which does not change the nutritive value of the product in most cases and enhance the texture, flavour, colour, appearance and elongate the shelf life. Also, processing can help the grain digestion and absorption by the human body(232).

Important whole grains consumed in the western diet include wheat, oats, rice, maize, corn, rye and barley (226, 233). Increased consumption of wholegrain is linked to reduced risk of chronic diseases including CVD (234-236), type-II diabetes(237, 238) ,some cancers(239) body weight (240) and gastrointestinal disease (241). Although recommended daily intakes of whole grains are not clearly defined and vary between countries, the 2010 Dietary Guidelines for Americans (242) recommended 6-11 servings per day of grains (243) depending on person's energy needs (244).On the other hand, the NHS (172) eat well plate gives more general advice with stating the consumption of rice, potatoes, pasta, bread and other foods rich in starch as a third of a plate and promoting the consumption of whole grain products (223).

1.5.1 Wholegrain consumption and CVD risk

Evidence from observational studies suggests that increased consumption of less-refined wholegrains is associated with a reduced risk of CVDs. A series of large observational studies such as Iowa Women's Health Study (IWHS), the Nurses' Health Study, Norwegian County Study (NCS) and the Physician's Health Study, established the beneficial effects of wholegrains on CVDs (172, 188, 230, 235, 236, 245, 246). For example, in the IWHS trial, an inverse association between wholegrain intake and risk of death from ischemic heart disease was observed after adjustment to potential confounding factors and total dietary fibre intake in 34,492 postmenopausal women aged 55-69 y (226, 235). Moreover, a strong inverse association between wholegrain intake and risk of coronary heart disease was observed in 75,521 women aged 38-63 y in the highest quintile of whole grain consumption compared with those in the lowest quintile (226, 236). In addition to these large prospective studies, recent systematic reviews and meta-analysis are also suggesting the same link between wholegrain consumption and CVDs. For example, Ye et al. performed a meta-analysis to assess findings from 10 prospective cohort studies in the area and suggested a 21% reduction in CVD risk with highest category of intake(3-5 serving/d, 44.4 g/d) of wholegrains when compared to lowest category (rare/never consumers) after adjustment for known risk factors (247). The same study also assessed the dietary fibre intake in relation to CVDs in 15 prospective cohort studies and stated that greater consumption of fibre was significantly and inversely associated with CVDs, although there were substantial heterogeneity across studies (247). A dose-response meta analyses carried out by Aune et al, suggested reductions in the relative risk per 90g/day for coronary heart disease and cardiovascular disease, 19% and 22% respectively and for the highest versus lowest category of wholegrain intake, 21% and 16% respectively(15). Although the consistent findings from observational studies suggest a cardio protective effects of a diet rich in wholegrains, the limitations of these studies such as uncontrolled confounders, inaccurate and different methods of measurements (including food-frequency questionnaires) should be considered and the proposed evidence should be supported with well controlled and long-term RCTs (248).

Intervention studies that have been carried out in relation to the cardiovascular health benefits of wholegrains are less consistent but still majorly report the positive impact of wholegrain consumption. A meta-analysis of 24 RCTs assessed the effect of daily whole-grain dose ranged from 28g to 213 g in comparison to a background diet low in wholegrains on blood lipid biomarkers. The duration of the studies ranged from 2wk to 16wk, mostly lasted 6-8wk and 16 out of 24 studies included hypercholesteraemic subjects with mean baseline concentrations >5.2mmol/L. This study found out that wholegrain consumption may reduce total and LDL cholesterol by 0.09 and 0.12 mmol/L, respectively (249). The study especially highlighted a causal link between grains high in viscous B-glucans-found mainly in oat and barley and lower total and LDL cholesterol (249). Another meta-

analyses of 21 RCTs investigated the effects of wholegrains on metabolic risk factors and reported significantly lower concentrations of fasting glucose, total and LDL cholesterol.(250) The inconsistency of the results obtained from other RCTs might be due to different study designs and incorporation of different types of wholegrain foods within the intervention diet.(231)

1.5.2 Oat Structure and Nutritional Composition

Oats (*Avena sativa*) are member of the wholegrain family with a nutritional profile that exert health benefits (251). The morphology of the oat grain is shown in **Figure 1.7** (252). The outer hull is tightly attached to the oat groat which mainly consists of bran, germ and endosperm (253). The bran that underlies the hull is acting like an envelope to the rest of the groat and is composed of the outermost pericarp, seed coat, aleurone and sub-aleurone layer (253). The aleurone layer of the bran is particularly rich in phenolics, minerals, vitamins and also contain cell wall polysaccharides including cellulose, arabinoxylan and β-glucan. Starchy endosperm mainly contains β-glucan that is metabolically inactive but supplies nutrients to the growing embryo during germination (252, 253). Thick cell walls that are resistant to digestion surround aleurone and subaleurone cells whereas thin cell walls cover endosperm(252). The germ is the metabolically active part in the germination process and transports nutrients from the starchy endosperm to embryonic axis (253).

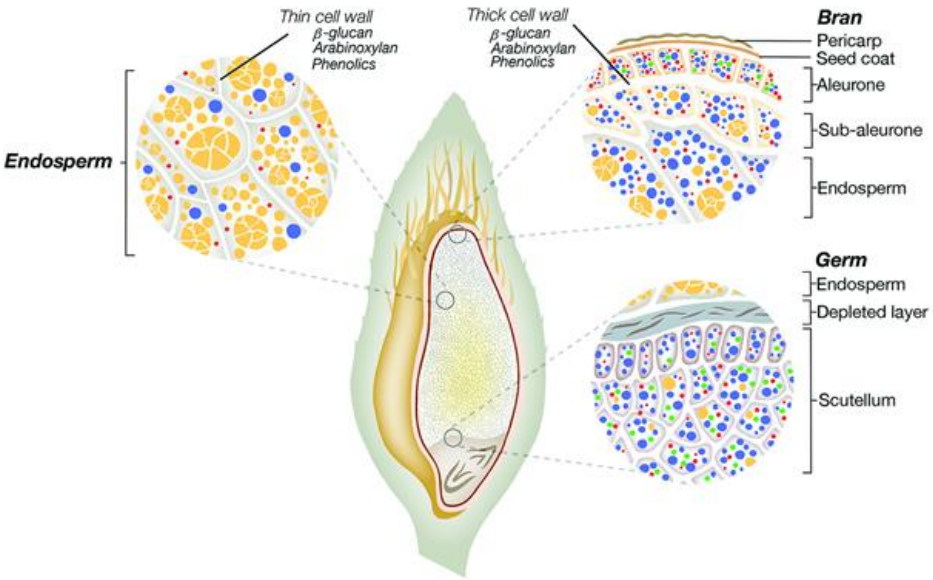


Figure 1.7 Schematic representation of the oat grain. Different oat tissues (bran, germ and endosperm) are shown in higher magnifications together with nutrients they contain. Figure adapted from Grundy et al, 2018(252).

As with other cereals, oats are a good source of slowly-digestible starchy carbohydrates, protein, vitamins, minerals and soluble fibre. **Table 1.2** gives an overview of macro and micronutrient content of oats with contribution of each nutrient to the total dry matter in % and where they are located within the plant structure. Wholegrain oats are typically very rich in dietary fibre, constituting 20-37% of hulled and 10-12% of de-hulled oats (254). Fibre can be classified as into soluble and insoluble fractions, forming 40% and 60% of the total oat fibre, respectively (254). Insoluble oat fibre is mainly cellulose, hemicellulose and lignin whereas soluble fibre is predominantly coming from β-glucan (254). Oat β-glucan is linear, unbranched polysachharide composed of 1-4-*O*-linked (70%) and 1-3- *O*- linked (30%) B-D-glucopyranosyl units (255). In addition to dietary fibre, oats are also considered as a source of phytochemicals including tocols, phenolics and avenanthramides (26, 256).

Table 1.2 Nutritional Composition of Oats.

Contribution of macro and micronutrients in % to the total dry matter and the location within the plant structure.

Component of Oat	Availability in Oat (%)	Location in the plant structure	References
Starch	Amylose:60 %	Endosperm	(257-260)
Protein	Total:11-15 % Globulins: 80 % of total protein Prolamins: 15% of total protein Glutelin: 5-66 % of total protein Albumin: 1-12 % of total protein	Germ and bran	(261, 262)
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	(21, 263)
Trace Minerals	Calcium: 0.54% Iron: 0.047%	Bran	(264)
Vitamins	Niacin: 0.032% Thiamine:0.002% Riboflavin:0.001%	Bran	(264)
Fibre	Total: 10-12% β-glucan : 2.3–8.5 %	Bran	(254, 265)
Phytochemicals	**α-Tocotrienols and α-tocopherols**: 86-91% of total tocols **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	(266, 267)

1.5.3 Oat Consumption and CVD Risk

Epidemiological studies provide strong protective evidence of wholegrain and dietary fibre intake on CVDs as discussed in the previous section (268-270). However, it is difficult to assess the association between oats and CVDs specifically from these studies and there are not any epidemiological studies focusing solely on oats. On the other hand, evidence accumulating from RCTs strongly suggest that regular consumption of oats, particularly β-glucan is lowering CVD risk markers (58, 271). To highlight the findings of some meta analyses in the area, Whitehead et al. showed that consumption of at least 3g of oat β-glucan per day is associated with decreased levels of total cholesterol (0.25mmol/L) and LDL (0.30mmol/L) relative to control without changing HDL cholesterol and triglycerides in lean, overweight, or obese subjects with or without type 2 diabetes (271). Thies et al. also highlighted that the regular consumption of oats, even when containing less than 3 g β-glucan, has a beneficial effect on total and LDL-cholesterol, particularly in hypercholesteraemic subjects(25, 272). Although the form of oat intervention differed between oat bran, wholegrain oats or oatmeal in the studies, Thies et al mentioned that this fact did not affect the outcomes(25).Most recently, Ho et al. further supported the dose dependent intake of β-glucan for decreasing LDL-cholesterol, non-HDL cholesterol and ApoB in middle aged subjects(58). The strong evidence about β-glucan led to health claims on cholesterol lowering properties of oats and their contribution to heart health, approved by FDA and EFSA (120, 273).

Additionally, oat consumption is reported to improve insulin sensitivity and post-prandial glycaemic control which may be explained by delayed glucose absorption due to high viscosity of β-glucan in the upper gastrointestinal tract (274, 275). Finally, there are limited number of RCTs examining the impact of oat consumption on BP and endothelial function. While the evidence regarding to BP is still inconsistent, studies measuring endothelial function by FMD did not report any changes following the chronic oat intake in healthy or hypercholesteraemic subjects(25, 276-279). However , a study testing the intake of wild green oat extract (1500mg/day) for 12 weeks found significant improvements in FMD in healthy older adults(280).

1.6 Conclusions

In conclusion, there is substantial amount of data emerging from epidemiological, clinical and *in vitro* studies that suggest cardio protective effects of polyphenols in humans. While flavonoids are demonstrating the great body of evidence, their circulating metabolites as phenolic acids or phenolic acid containing foods have also shown to be exerting the health benefits on vascular measures through NO related mechanisms. Oats are a source of phenolic acids and avenanthramides, however, most of the studies to date have predominantly centred on β-glucan without being controlled for their phenolics content and suggested cardiovascular benefits. Considering the evidence from polyphenol studies, oat phenolics may also have similar vascular health benefits or they may act synergistically with fibre to exert their health benefits. Therefore, more human studies are necessary to address the gap and understand the role of oat phenolics in defining cardiovascular benefits of oats.

1.7 Hypothesis and Study Objectives

The hypothesis of this thesis is that consumption of oats containing phenolic acids and avenanthramides will be effective at inducing beneficial improvements in human circulatory function, through the influence of absorbed small phenolic acids on endothelial function, microvascular function and blood pressure and lead to a healthy cardiovascular system.

Objectives of the thesis are as follows:

- To determine phenolic acid and avenanthramide composition and content of different varieties of commercial oats to see the variation in the levels of phenolic content and to estimate the amounts delivered to the consumer in a portion of oats. This objective was set to inform the following clinical work to provide an achievable dietary intake level of these compounds in intervention products.

- To examine effects of acute (i.e. 1-24h post-intake) consumption of oats containing phenolic acids and avenanthramides on markers of CVD risk relative to an energy matched control intervention in pre and stage one hypertensive men, with a randomized controlled trial.

- To examine the dose-dependent effect of 4 weeks of daily consumption of soluble fibre matched meals providing either a high dose or a moderate dose of oat phenolic acids and avenanthramides on markers of CVD risk relative to an energy and soluble fibre matched control in pre and stage one hypertensive men and women, with a randomized controlled trial.

Chapter 2 Composition and Content of Phenolic Acids and Avenanthramides in Commercial Oat Products: are Oats an Important Polyphenol Source for Consumers?

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Food Chemistry: X, doi.org/10.1016/j.fochx.2019.100047

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2.1 Abstract

Oats contain a range of phenolic acids and avenanthramides which may have health benefits. Analysis of 22 commercial oat products (oat bran concentrate, oat bran, flaked oats, rolled oats and oatcakes) using HPLC-DAD detected eleven bound and thirteen free + conjugated phenolic acids and avenanthramides. The oat products (excluding concentrate) provided between 15.79 to 25.05 mg total phenolic acids (9.9 to 19.33 mg bound, 4.96 to 5.72 mg free +conjugated) and between 1.1 and 2 mg of avenanthramides in a 40g portion while an 11g portion of oat concentrate provided 16.7 mg of total phenolic acids (15.17 mg bound, 1.53 mg free + conjugated) and 1.2 mg of avenanthramides. The compositions and concentrations of the components in the different products were broadly similar, with the major component being ferulic acid (58-78.1%). The results show that commercial oat products are a source of phenolic acids and avenanthramides for consumers.

Key words

Oats, avenanthramides, phenolic acids, ferulic acid, oat products, oat cakes, oat bran

2.2 Introduction

Epidemiological evidence suggests that regular consumption of whole grains is associated with a lower risk of chronic diseases, such as cardiovascular disease (CVD), type 2 diabetes and certain cancers(281).Meta-analyses of multiple human intervention studies provide strong evidence that regular oat intake lowers blood cholesterol, improves insulin sensitivity and post-prandial glycaemic control (58, 275). Oats (*Avena sativa)* are wholegrain cereals which provide proteins, unsaturated fatty acids, vitamins, minerals (282), phenolics (including avenanthramides that are unique to oats), and significant quantities of dietary fibre, including cellulose, arabinoxylan and β-glucan (21). There is a solid body of evidence and a health claim approved by EFSA approving the cholesterol lowering properties of oat β-glucans. (20, 58, 271).

A diet rich in oats can provide phenolic acids and avenanthramides that may contribute to the beneficial health effects. Although there are mixed findings from different studies testing different foods, well designed randomized controlled trials investigating a variety of foods such as coffee and blueberries showed protective effects of circulating phenolics against CVD (29, 30, 283-285). Avenanthramides have also been shown to be bioavailable in humans and may exert anti-inflammatory, anti-proliferative and vasodilatory effects to protect against CVD and colon cancer (27, 119, 165, 286, 287).

Phenolic acids comprise one aromatic ring bearing an acid group and one or more hydroxyl groups (288) and are present in the oat grain in three forms: 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, notably cell wall polysaccharides (dietary fibre and lignin) (289). Cereal phenolic acids consist of two major types: based on either hydroxycinnamic acid or hydroxybenzoic acid. Hydroxybenzoic acids in oats include protocatechuic, syringic, vanillic , *p*-hydroxybenzoic and gallic acids, while hydroxycinnamic acids in oats are ferulic, *p*-coumaric, *o*-coumaric, caffeic and sinapic acids (**Figure 1.5**) (26). Avenanthramides consist of an amide conjugate of anthralinic acid and hydroxycinnamic acids. The 3 major types are esters of 5-hydroxyanthranilic acid with p-coumaric (2p aka A), and ferulic (2f aka B), and caffeic (2c aka C) acids which occur predominantly in the bran layer of the oat grain (**Figure 1.5**) (290, 291).

Several previous studies have reported the presence and amounts of phenolic acids and avenanthramides in oats (115, 267, 292, 293). However, some of these studies analysed experimental material (including hulled grains) which would not be available to consumers while others analysed either avenanthramides or phenolic acids but not the two groups together. Therefore, the aim of this study was to determine the composition and amounts of phenolic acids and avenanthramides in a number of commercial oat samples, determining differences between products (oat bran, oat bran concentrate, rolled, flaked oats and oat cakes) and estimating the amounts consumed in typical portions of oat-based products in order to understand their

contribution to dietary intakes of phenolics and to inform further clinical trials aiming to test the bioactivity of these compounds at normal intake levels.

2.3 Materials & Methods

2.3.1 Chemicals and reagents

All individual phenolic acid and avenanthramide standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC column and guard cartridges were obtained from Phenomenex (Torrance, CA, USA). Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich or Fisher Scientific.

2.3.2 Oat Samples

22 commercial oat products were purchased in local shops or online and comprised 4 oat brans, 5 oat flakes, 6 rolled oats, 5 oatcakes and one oat concentrate (**Table 2.1**). The samples were milled using a coffee grinder and kept at -20 °C until analysis to protect bioactive ingredients from degradation.

Table 2.1 Brands, names, types and numbers of commercial oat products.

Brand	Type	Number
Oatwell	Bran concentrate	1
White's (Fine cut)	Bran	2
Mornflake	Bran	3
Sante (Otreby Owsiane)	Bran	4
Greenlands (organic)	Bran	5
M&S (Traditional British porridge oats)	Flaked	6
Sainsbury's (SO organic Scottish oats)	Flaked	7
Sainsbury's (Scottish porridge oats)	Flaked	8
Flahavan's (Irish organic jumbo oats)	Flaked	9
Kupiec (plain)	Flaked	10
Quaker (Oats so simple original-sachets)	Rolled	11
Sainsbury's (Taste the difference Scottish)	Rolled	12
Asda (Original porridge)	Rolled	13
Quaker (Jumbo oats)	Rolled	14
Quaker	Rolled	15
Scott's (Porridge oats)	Rolled	16
Nairns (Fine milled, 85% oats)	Oatcake	17
Nairns (Organic, enriched with oatbran, 85% oats)	Oatcake	18
Savour Bakes (Scottish rough, 89% oats)	Oatcake	19
Clearspring (Organic, 83% oats)	Oatcake	20
Paterson's (Rough, % *ng)	Oatcake	21
Stockan's (Orkney, thin, 76% oats)	Oatcake	22

* ng (not given); proportion of oat (%) is not provided on the package of the product.

2.3.3 Extraction of free, conjugated and bound phenolic acids

Phenolic acids and avenanthramides were extracted in two separate fractions (i.e. free + conjugated and bound) (**Figure 2.1**), in three replicates, using the method of Schär et al (152). 2 ml of hexane were added to 20.0 ± 0.1 mg of milled oat samples for defatting. Solutions were vortexed for 30 seconds, sonicated for 10 min, vortexed and kept on a shaker for 50 min. Following centrifugation for 15 min at 5000 rcf, the supernatant was discarded and the wet samples were dried with a Savant™ SPD131DDA SpeedVac™ Concentrator (ThermoFisher Scientific, Waltham, MA, USA) for 10 min. The first extraction step was carried out by adding 80:20 ethanol/water (1mL) and samples were vortexed until all of the flour was suspended. 0.375µg of internal standard (3-5,dichloro-4-hydroxybenzoic acid) was added and the samples were extracted for 10 min by sonication in a sonic bath. Samples were then heated to 80°C for 15 min and centrifuged for 15 min at 5000g. The supernatant was removed into a test tube and evaporated with a SpeedVac. This extraction was repeated twice without the heating and the addition of internal standard. The combined supernatants were evaporated to dryness. For the extraction of free + conjugated fractions, the dried supernatant was hydrolysed with 2M NaOH (400µl) with 10µM EDTA and 1% (w/v) ascorbic acid for 4 h and then acidified with 12 M HCl (125µl) to pH 2 and kept at -20 °C overnight. For the extraction of the bound fraction, the pellet was dried in a Speedvac for 5 min, 30 µg internal standard added and hydrolysis was carried out with 2 M NaOH solution (800µl) , 10µM EDTA and 1% (w/v) ascorbic acid for 16-18h with gentle agitation. Following hydrolysis, samples were centrifuged for 15min at 16100g, supernatants were transferred to clean Eppendorf tubes and acidified with 12 M HCl (220 µl). Bound and free + conjugated fractions were extracted with ethyl acetate (3 x 800 µL), evaporated to dryness and re-dissolved in 2% (v/v) aqueous acetic acid (100 µL) using vortexing for 30s, sonication for 1 min and shaking for an hour. Final solutions were centrifuged for 5min at 16100g and transferred to vials containing a low volume insert for HPLC analysis.

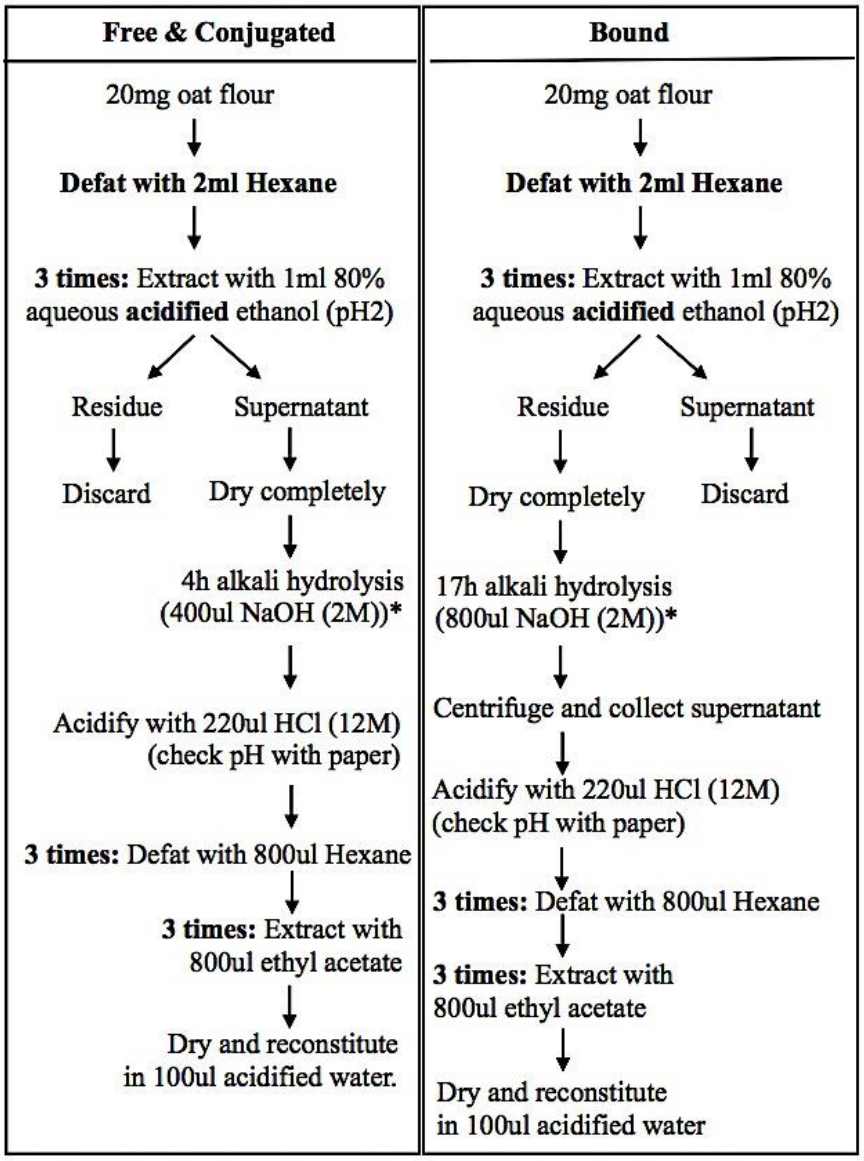

Figure 2.1 Flow chart illustrating the extraction of phenolic acids and avenanthramides from free, conjugated and bound fractions of oats. Adapted from Li et al, 2010(294).

2.3.4 HPLC Analysis

Phenolic acids were identified and quantified using an Agilent 1100 series HPLC (Agilent Technologies Ltd, Santa Clara, CA, USA) equipped with a quaternary pump, autosampler, column thermostat, sample thermostat and photodiode array detector. Phenolic acids were separated on a Kinetex biphenyl column (100A 250x4.6 mm length, 5µM particle size; Phenomenex) using a gradient elution programme developed in house for the separation of 18 different phenolic acids and 3 avenanthramides. Mobile phase A consisted of 0.1% (v:v) formic acid in HPLC grade water (A), while mobile phase B was 0.1% (v:v) formic acid in methanol. The following optimised gradient protocol was run: 0 min, 95% A; 20 min,75% A; 25min,74% A; 30 min, 65% A; 40 min,64% A; 53 min, 30% A; 56 min, 5% A; 61 min, 5% A; 62 min, 95% A; 65 min, 95% A. The flow rate of the mobile phase was 1.0mL/min, the column temperature was 30°C and the sample injection volume was 20µl. Quantification of phenolic acids and avenanthramides were made at 280 nm. The retention time and absorbance of each compound was established by running standards (**Table 2.2**). All standards were prepared as stock solutions at 1mg/mL in methanol and stored at -80°C. Phenolic acids and avenanthramides were quantified by calculating the ratio of the area of the analyte to the area of the internal standard (i.e. 3-5,dichloro-4-hydroxybenzoic acid). Quantification was based on 10 point linear calibration curves of phenolic acid and avenanthramide standards which covered the range present in the oat samples and had R^2 values above 0.9 (**Table 2.2**). The concentrations of individual components were stated as µg/g. Good reproducibility was observed between replicate analyses with an average of 9% for the coefficient of variation for all samples. All data were analysed using Agilent Chem Station software.

Table 2.2 Identities (compound), retention times (RT), molecular weights (MW) and peak absorbances (PA) of phenolic analytical standards determined by HPLC-DAD and R2 values of identified compounds.

Compound	RT (min)	MW	PA (nm)	R^2
Gallic acid	5.4	170.1	218	-
4-Hydroxybenzoic acid	15.1	138.1	255	0.997
2,4-Dihydroxybenzoic acid	17.2	154.1	255	-
4-Hydroxyphenyl acetic acid	18.4	152.2	235	-
Caffeic acid	21.0	180.2	324	0.995
Vanillic acid	23.0	168.2	218	0.986
4-Hydroxybenzaldehyde	23.7	122.1	224	0.997
Homovanillic acid	24.5	182.2	280	-
Syringic acid	28.3	198.2	220	0.940
p-Coumaric acid	30.5	164.2	310	0.995
Vanillin	33.0	152.2	280	0.996
Salicylic acid	33.7	138.1	238	-
Ferulic acid	36.8	194.2	323	0.995
Syringaldehyde	38.6	182.2	218	-
Sinapic acid	39.8	224.2	320	0.998
3-5,Dichloro-4-hydroxybenzoic acid	40.5	207.0	217	-
o-Coumaric acid	41.2	164.2	278	-
Avenanthramide C	47.1	315.3	340	0.980
Avenanthramide A	50.5	299.3	320	0.943
Avenanthramide B	52.0	329.3	338	0.965

2.3.5 Statistical Analysis

Means and standard errors of 3 replicates were calculated for each sample. Data were quantified and expressed as μg/g fresh weight. Calibration curves of phenolic acid and avenanthramide standards were created by MS Excel 2010.

2.4 Results and Discussion

2.4.1 Identification of Phenolic Acids and Avenanthramides in Commercial Oat Products.

Standards of twenty different phenolic compounds (including phenolic acids and avenanthramides) which could be present in oat products were analysed by HPLC-DAD (**Figure 2.2 A**), measuring their absorbance maxima and retention times (**Table 2.2**). Comparisons with these standards identified eleven components in the bound fractions and thirteen in the free + conjugated fractions extracted from the products. This is illustrated by the typical chromatograms for oat bran (product 3) in **Figure 2.2 B** and **C**. While both the bound and free + conjugated fractions contained 4-hydroxybenzoic acid, vanillic acid, caffeic acid, 4-hydroxybenzaldehyde, syringic acid, p-coumaric acid vanillin, ferulic acid and sinapic acid, the three avenanthramides (avenanthramide A, avenanthramide B and avenanthramide C) were only present in the free + conjugated fractions.

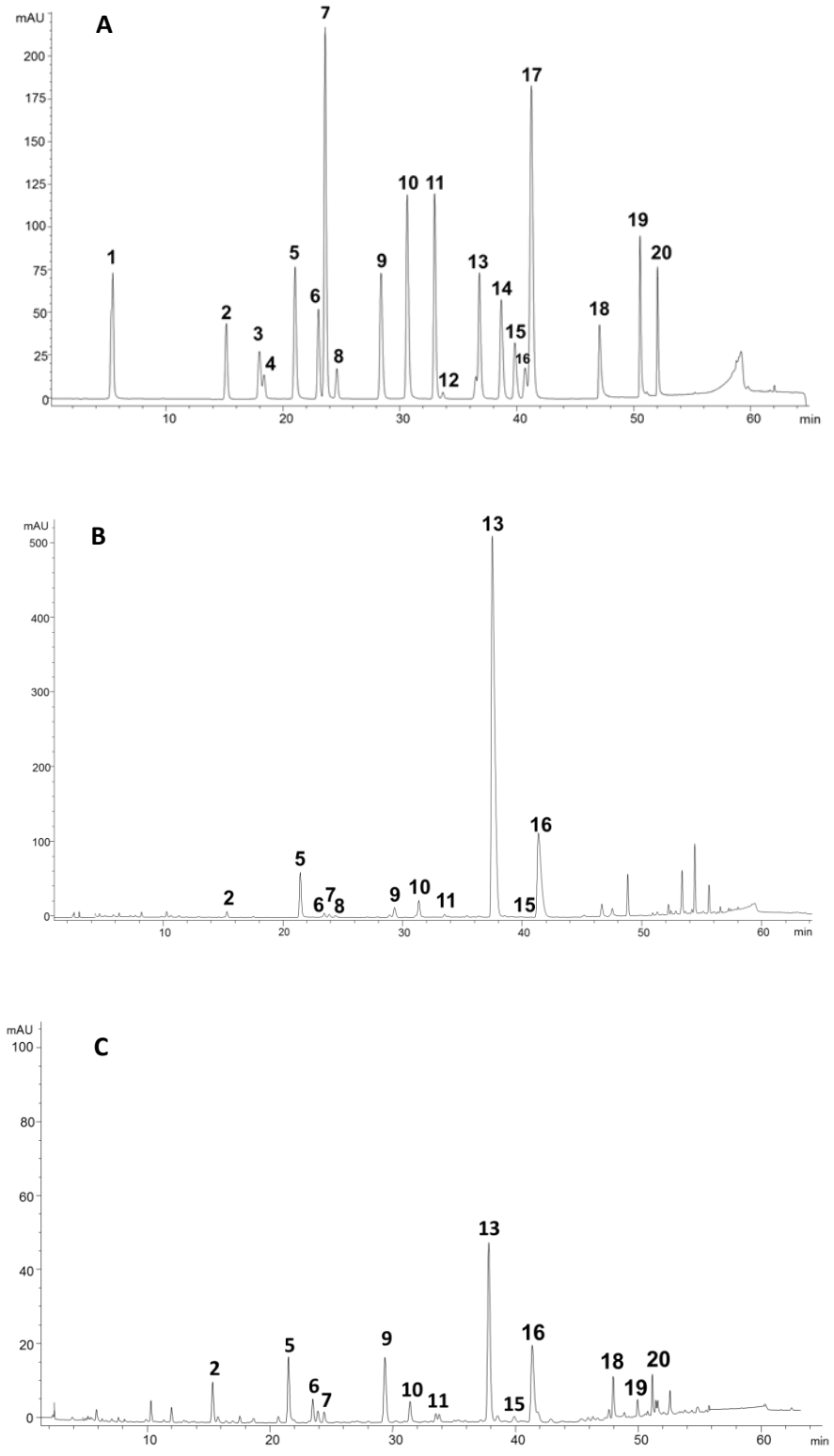

Figure 2.2 Chromatogram of analytical phenolic acid standards at 280nm. (A) Representative chromatograms of oat sample 3 for bound phenolic acids (B) and free &conjugated phenolic acids at 280nm (C).Compounds were identified based on retention time and absorbance spectrum(Table 2.2): 1, gallic acid; 2 , 4-hydroxybenzoic acid; 3, 2,4-dihydroxybenzoic acid; 4, 4-hydroxyphenyl acetic acid; 5, caffeic acid; 6, vanillic acid; 7; 4-hydroxybenzaldehyde ; 8, homovanillic acid; 9, syringic acid; 10, p-coumaric acid ; 11,vanillin ; 12,salicyclic acid; 13, ferulic acid; 14, syringaldehyde; 15, sinapic acid; 16, 3-5,dichloro-4-hydroxybenzoic acid; 17, o-coumaric acid; 18, avenanthramide C; 19, avenanthramide A; 20, avenanthramide B.

2.4.2 Total Contents of Phenolic Acids and Avenanthramides.

The mean total contents of phenolic acids and avenanthramides in the groups of oat products was highest for the oat bran concentrate (1518.6 μg/g) followed by oat bran (626.3±60.1 μg/g), flaked oats (438.1±27.0 μg/g), rolled oats (415.8±14.9 μg/g) and oatcakes (394.8±24.2 μg/g) (**Figure 2.3 A**). All the results are reported on a fresh weight (FW) basis. The bound phenolic acid fraction made the greatest contribution to the total phenolic contents of all products (62.7-90.8%) while the amounts of the free + conjugated fractions were lower in all products (9.2-37.2%). This is in agreement with other studies (295, 296). When the contents and compositions of phenolic acids and avenanthramides were compared within each group of oat products, the bran products showed the greatest variation in both free + conjugated (ranging from 100.9 to 194.9 μg/g) and bound (ranging from 359.5 to 592.4 μg/g) fractions, the free + conjugated fraction also varied between the samples of oat flakes (115.7 -210 μg/g). Oat bran concentrate comprises mainly fibre and, in agreement with the literature, our analysis showed that this fraction was rich in bound phenolic acids (266). The total contents of phenolic acids in the oat bran products were only slightly higher than those in the flaked oats, rolled oats and oatcake products. Similar results for different oat products were reported by Matilla et al (2005) who suggested that the similar phenolic content of oat bran might be due to the processing method which results in the bran containing some aleurone and starchy endosperm as well as outer layers of the grain (pericarp and testa) (267, 297).

Avenanthramides were only present as soluble forms with the mean total concentrations being highest in oat bran (49.6±8.3 μg/g) followed by flaked oats (48.8±10.3 μg/g), oatcakes (43.8±6.9 μg/g), oat bran concentrate (30.6 μg/g) and rolled oats (28.3±2.9 μg/g) (**Figure 2.3 C**). Avenanthramides have been reported to be heat stable under conditions of commercial processing and the levels of these compounds in oatcakes, which are mostly processed products in our sample range, were higher than in the oat bran concentrate which contained highest total levels of phenolic acids (298). The concentrations determined in this study were broadly consistent with those reported previously (115, 267, 297, 299). However, whereas Chen et al. (2018) and Pridal et al. (2018) reported higher concentrations of avenanthramides in oat bran samples in comparison to whole oats, including rolled oats, Mattilla et al (2005) reported that commercial oat bran products produced by conventional milling techniques could not be discriminated from oat flakes based on their avenanthramide content, which is in agreement with our results. It should also be noted that the contents of phenolic acids and avenanthamides in oat grain will vary with the crop genotype and the growth conditions (including location and environment) (299, 300).

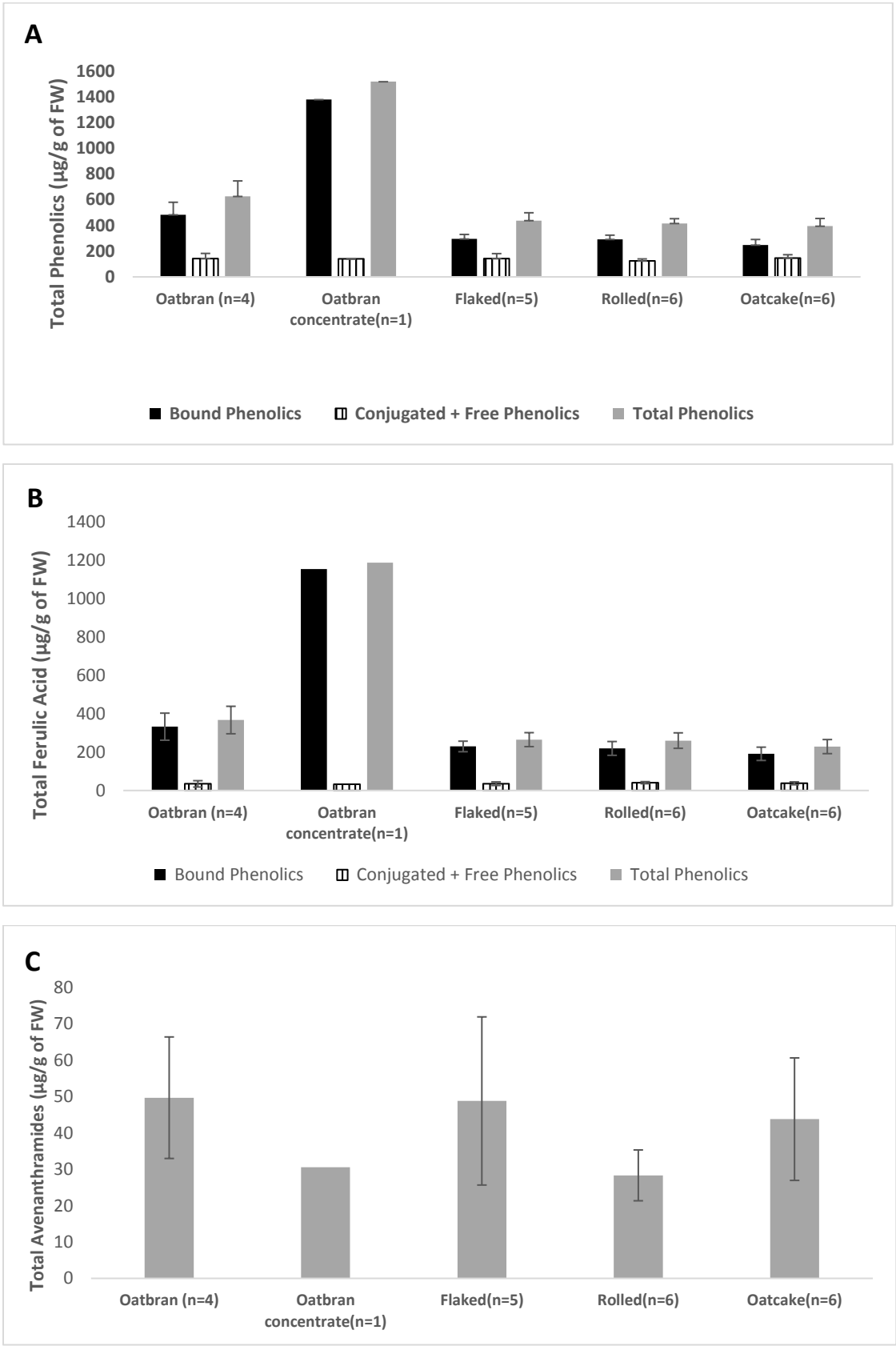

Figure 2.3 Comparison of total, conjugated+free and bound phenolic acid levels(A), ferulic acid (B) and total avenanthramides(C) between oat products including oatbran , oatbran concentrate, flaked oats, rolled oats and oatcake. Data are expressed as the mean ± SEM. n indicates the number of each product analysed.FW= fresh weight.

2.4.3 Compositions and Individual Contents of Phenolic Acids and Avenanthramides.

The most abundant bound phenolic acids in all oat products analysed were ferulic acid, caffeic acid and sinapic acid (**Table 2.3**). The oat bran concentrate had the highest concentrations of these compounds in the bound fraction (1153.6 μg/g, 72.2 μg/g and 80.3 μg/g, respectively) whereas oatcakes had the lowest (191.1±14.2 μg/g, 11.9±0.9 μg/g and 21.7±1.1 μg/g, respectively). Ferulic acid, sinapic acid and avenanthramide B were the major components in the free + conjugated fractions of all products, with rolled oats having the highest concentration of ferulic acid (40.8±2.2 μg/g), oat bran concentrate the highest concentration of sinapic acid (36.2 μg/g) and oat bran the highest concentration of avenanthramide B (27.6 ±4.6μg/g).

The compositions of oat phenolics reported in other studies are largely in line with our findings, but there are few inconsistencies. Ferulic acid was reported to be the major component in both fractions by other workers (26, 295, 301). However, *p*-coumaric acid has also been reported to be highly abundant in other studies whereas the concentrations in our study were low (26, 302, 303). In agreement with Chen et al (2018), avenanthramide-B was the predominant avenanthramide. However, the concentrations of avenanthramide-C reported here are lower than in other studies (26, 295). These inconsistencies may be due to differences in samples used (e.g. the samples analysed by Shewry et al (2008) contained the hulls which are rich in *p*-coumaric acid), the methods used for extraction and analysis, and genetic and environmental factors.

Table 2.3 Composition and contents of phenolic acids and avenanthramides in individual oat products.

Concentration (μg/g of FW)[a]	Bran Conc. (1)	Bran (2)	Bran (3)	Bran (4)	Bran (5)	Flaked (6)	Flaked (7)	Flaked (8)	Flaked (9)	Flaked (10)	Rolled (11)
4-hydroxybenzoic acid											
Free &Conjugated	7.1± 0.1	4.1± 0.2	9± 0.1	3.67± 0.2	3.3± 0	4.6± 0.2	3.6± 0.1	5.1± 0.1	4.3± 0.1	4± 0	6.3± 0.3
Bound	15.5± 0.1	4.5± 0.2	8.5± 0.4	12.3± 0.7	10.7± 0.8	3.9± 0.4	3.2± 0	1.9± 0.2	3.5± 0.3	5.6± 0.2	3.9± 0.1
Vanillic acid											
Free& Conjugated	5.5± 0	6.8± 0.2	10.7± 0.2	4± 0.6	4.5± 0.2	7.1± 0.4	9.5± 0.4	8.1± 0.2	6.3± 0.4	3.5± 0.1	9.6± 0.6
Bound	12.9± 0.3	6.9± 0.1	12.2± 0.3	16.9± 1.2	15.3± 1.1	5.6± 0.4	6.5± 1.4	9.2± 1	6.5± 0.5	3.4± 0.2	5.8± 4.2
Caffeic acid											
Free& Conjugated	8.8± 0.1	1.1± 0.2	1.9± 0.1	19.3± 0.9	19.4± 0.4	1.4± 0	1.5± 0	1.9± 0	27.1± 0.3	11.8± 0.3	1.8± 0.1
Bound	72.7± 0.7	21.1± 0.9	33.2± 0.3	39± 3.8	32.5± 2.6	15.4± 0.4	17.9± 0.6	13.1± 0.8	15± 0.7	17± 0.1	17.4± 1.2
4-Hydorxybenzaldeyhde											
Free& Conjugated	2.6± 0	2.8± 0.1	3.8± 0	2.3± 0.1	2.5± 0	2.7± 0	3.5± 0.1	3.1± 0	3± 0.2	2.3± 0.1	3.6± 0.1
Bound	4.1± 0.1	2.2± 0.1	3.6± 0.2	4.5± 0.2	4.1± 0.2	1.6± 0	1.6± 0.1	1.4± 0	2.3± 0.1	1.6± 0	2.9± 0.1
Syringic acid											
Free& Conjugated	10.7± 0.1	6.8± 0.9	13.1± 0.2	6.8± 0.3	8.6± 0.1	7.5± 0.5	8.5± 0.4	11.9± 0.1	10.7± 0.1	8.2± 0.3	10.2± 0.7
Bound	18.2± 0.4	5.1± 0.7	15.2± 0.7	20.8± 2	21.1± 1.8	5± 0.6	6.4± 0.1	5.2± 0.1	7.2± 0.4	6.8± 0.2	6.7± 1
P-coumaric acid											
Free& Conjugated	2.3± 0	6.3± 0.5	6.8± 0.5	2.1± 0.1	2.1± 0.1	5.5± 1.4	3.1± 0.2	4.3± 0.1	3.3± 0.1	2.3± 0.1	4.1± 0.3
Bound	17.5± 1.3	49.4± 3.9	36.6± 32.7	5.7± 0.7	5.8± 0.9	6.8± 1.5	13.7± 5.2	3.5± 0.5	5.3± 0.3	2.7± 0.2	5.4± 0
Vanillin											
Free& Conjugated	2.8± 0	nq	2± 0	2.7± 0	2.7± 0	nq	nq	nq	3.5± 0.2	2.7± 0	nq
Bound	4.5± 0.4	3.3± 0.1	3.9± 1	3± 0.2	2.4± 0.1	2.3± 0.1	2.2± 0	2.3± 0.4	2.5± 0.1	2± 0.2	2.1± 0.1
Ferulic acid											
Free& Conjugated	32.8± 0.1	32.8± 2.8	58.6± 0.1	24± 1.9	23.1± 0.9	34.1± 0.8	40.8± 2.6	38.1± 0.7	44.2± 1.2	20.2±0.8	50.6± 3.5
Bound	1153.6± 5.8	247.2± 8.2	348.7±3.3	416.9±29.7	317.1±10.2	230.1±10.3	255.7±3.6	222.7±7.9	251.8±3.1	187.2±6.2	262.7±4.7
Sinapic acid											
Free& Conjugated	36.2± 0.1	14.2± 0.8	32 ± 0.2	15.4± 1.1	17.1 ± 0.5	19.9 ± 0.4	21.7 ± 1.4	26.3± 0.4	25.9 ± 0.5	15.4±0.1	8.7 ± 0.6
Bound	80.3± 1.3	20.6± 0.6	50.2± 0.4	74.1± 4.9	60± 3.3	23.3± 1.9	23.1± 0.7	19.5± 3.5	32.8± 1.6	20.5±1.2	25.7± 2.1
Avenanthramide-C											
Free& Conjugated	11.5± 0.2	2.2± 0.2	1.6± 0.2	11.6± 3.2	12.4± 1.8	1± 0.1	1± 0.1	1.4± 0.1	18.1± 0.4	18.5±8.5	8.3± 0.4
Avenanthramide-A											
Free& Conjugated	6.8± 0.2	8.5± 0.9	23.8± 4.7	12.9± 3.4	15.4± 2.4	14.9± 0.8	9.2± 1.3	15± 0.7	25.4± 0.8	18.7±4.9	6.7± 0.3
Avenanthramide-B											
Free& Conjugated	12.3± 0.3	15.3± 1.5	31.5± 1.5	26.6± 4.7	36.8± 6.9	25.7± 1.9	13.3± 2.5	18.7± 0.8	38.3± 1.5	24.5±3.7	7.3± 1.2
Total Free&Conjugated	139.3± 0.5	100.9±8.2	194.9± 2.6	129.5±14.1	146.8± 9.4	124.4± 5.2	115.7±8.3	134± 2.8	210± 1.9	130.3±15.8	117.2± 7.1
Total Bound	1379.3± 6.9	359.5± 14.8	512.2± 36.9	592.4± 43	469± 19.1	294± 13.7	330.2±2.8	278.8± 13.6	326.9± 3.7	246.7± 8.1	332.5± 13.1
Total Phenolics	1518.6±277.3	460.5± 58.3	707± 80.3	721.9±105	615.7± 72.2	418.2± 38.5	445.9±48.1	412.8± 35.8	536.9± 26.2	377± 27.2	449.7± 48.6

Table 2.4(continued) Composition and contents of phenolic acids and avenanthramides in individual oat products.

Concentration (µg/g of FW)[a]	Rolled (12)	Rolled (13)	Rolled (14)	Rolled (15)	Rolled (16)	Oatcake (17)	Oatcake (18)	Oatcake (19)	Oatcake (20)	Oatcake (21)	Oatcake (22)
4-hydroxybenzoic acid											
Free &Conjugated	3.1± 0.2	5± 0.1	5.4± 0.3	6± 0.3	5.2± 0.7	4.7± 0.1	4.5± 0.2	4.1± 0.1	3.9± 0.2	4.9± 0.5	3.5± 0
Bound	2± 0.2	2.7± 0.5	3.5± 0.5	3.7± 0.1	3.9± 0.2	3± 0.1	3.7± 0.3	2.5± 0.2	2.8± 0.2	3.6± 0.3	2.6± 0.3
Vanillic acid											
Free& Conjugated	8.6± 0.6	7.3± 0.2	7.2± 0.3	8.6± 0.3	7.8± 0.5	6.9± 0.1	8.3± 0.3	7.4± 0.2	7.5± 0.3	8.5± 0.6	5.6± 0
Bound	8.9± 1	5.8± 0.8	8.1± 1.2	6.4± 0.5	6.7± 0.4	6.3± 0.1	5.6± 0.1	5.8± 0.4	4.9± 0.4	7.6± 0.7	5.1± 0.4
Caffeic acid											
Free& Conjugated	1.4± 0	19.4± 0.6	1.5± 0	1.7± 0	1.8± 0.1	8.5± 0.3	11.5± 0.5	10± 0.3	12.8± 0.5	16± 1.2	12.8± 0.2
Bound	14.7± 1	12.1± 2.4	15.1± 0.6	18.4± 0.4	16.4± 0.3	11± 0.4	11± 0.7	10.4± 0.6	11.2± 1.2	16.4± 1.3	11.2± 1.2
4-Hydroxybenzaldeyhde											
Free& Conjugated	3.3± 0.1	2.9± 0	3± 0.1	3.4± 0.1	3.2± 0.1	2.9± 0	3.5± 0.1	3.3± 0	3.3± 0.1	3.5± 0.1	2.6± 0
Bound	1.5± 0.1	2.2± 0.2	2.3± 0.1	2.4± 0.1	2.4± 0.1	2.1± 0	2.1± 0.1	2.2± 0.1	1.9± 0.1	2.6± 0.1	1.8± 0.1
Syringic acid											
Free& Conjugated	7.7± 0.5	10.1± 0.4	8± 0.5	11± 0.7	9± 0.8	8.8± 0.2	11.8± 0.6	9.8± 0.2	12.2± 0.5	10.5± 0.8	7.3± 0.1
Bound	5.3± 0.7	4.7± 0.7	6.2± 0.7	7.8± 0.3	6.5± 0.5	4.5± 0.2	7.2± 0.7	4.9± 0.1	4.8± 0.4	5.9± 0.7	4.5± 0.5
P-coumaric acid											
Free& Conjugated	3.1± 0.3	3.5± 0.1	3.8± 0.1	4.4± 0.1	4.6± 0.2	2.9± 0.1	4.2± 0.2	2.9± 0.1	8.2± 0.3	4± 0.2	3.2± 0
Bound	3.5± 0.7	2.6± 1.7	3.9± 0.4	5.5± 0.6	5.7± 0.7	4.1± 0.2	8.5± 0.3	3.3± 0.8	5.6± 1.1	7.4± 1.6	1.9± 0.4
Vanillin											
Free& Conjugated	3.1± 0	4.6± 0.1	3.2± 0.1	3.5± 0.3	2.8± 0.4	4.3± 0.1	3.5± 0	3.2± 0.1	5.4± 0.1	3.6± 0.1	3.6± 0.1
Bound	2± 0.1	1.4± 0.7	3.5± 0.9	2.2± 0.1	2.1± 0.1	1.7± 0	1.8± 0.1	1.6± 0.1	1.4± 0.1	2± 0.1	1.4± 0.1
Ferulic acid											
Free& Conjugated	38± 1.9	37.3± 0.7	35.5± 2.6	41.1± 1.5	42.1± 3.3	32.4± 0.5	42.5± 1.9	34.8± 0.6	48.3± 1.5	40.1± 2.8	27.2± 0.5
Bound	238.5± 22.3	175.7± 33.3	177.3±21.1	243± 3.3	218.2± 8.3	181.7± 3.5	214.3± 10.4	198.9± 25.6	152.5± 17.9	242.7± 8.5	156.9± 16.1
Sinapic acid											
Free& Conjugated	17.4± 0.8	22.7± 0.5	17.9± 1.1	25.9± 1	15.2± 6.7	17.3± 0.4	22.9± 1	25.4± 1.3	21.9± 0.6	26.1± 2	16.1± 0
Bound	24.2± 2.4	23± 3.5	22.8± 2.7	28.6± 1.1	25.1± 1.4	19.9± 0.5	23.3± 1.7	22.8± 1	18.9±2.2	25.9± 2.6	19.6± 1.1
Avenanthramide-C											
Free& Conjugated	0.9± 0.1	9.4± 1.1	1.2± 0.1	1.5± 0.2	1.3± 0.3	5.8± 0.3	6.1± 0.9	10.7± 4.5	6.6± 0.4	23.3± 2.6	16.3± 4.2
Avenanthramide-A											
Free& Conjugated	7.8± 0.5	8± 0.2	11.4± 0.4	12.6± 0.6	16.8± 1.7	5.3± 0.1	6.5± 0.6	10.7± 2.5	13.8± 1.4	18.9± 1.2	14.6± 4.4
Avenanthramide-B											
Free& Conjugated	10.9± 0.6	12.3± 0.6	14± 0.9	19.1± 0.8	20.5± 3.1	11.5± 0.3	17.6± 1.8	23.7± 8.5	19.7± 2.2	26.5± 1.8	25± 10.4
TotalFree&Conjugated	103.3± 3.8	142.4± 0.8	112.2± 5.7	138.8± 4.1	130.2± 15.9	111.2± 1.9	142.9± 7.8	140.4± 7.7	163.5± 6.7	185.8± 13	137.7± 18.4
Total Bound	300.8± 28	270± 43.6	242.6±24.2	318± 5.8	287± 7.4	234.2± 4.5	277.5± 14.1	252.3± 28.5	203.9± 23.5	314.1± 14	205.1± 20.2
Total Phenolics	404.1± 45.9	412.4± 27.7	354.8±31.2	456.8±40.2	417.2±35.9	345.4± 27.6	420.4± 30.9	392.7± 28.3	367.4± 14.2	499.9± 29.9	342.8± 19.4

[a] Values are expressed as means± SEMs (n=3). nq=not quantified.

2.4.4 Multivariate Analysis of Phenolic Acid and Avenanthramide Composition.

Principal component analyses (PCA) of the phenolics present in the bound and free + conjugated fractions are shown in **Figures 2.4 and 2.5**, respectively. Panels **A, C** and **E** are PCA score plots which show the grouping of oat products based on their overall phenolic acid compositions while panels **B, D** and **F** are the corresponding loading plots which show the individual phenolic acids responsible for the separation. For the bound fraction, all three score plots showed clear clustering of the rolled oats, oatcake and flaked oats in the centre of the plot, and separation of the single oat concentrate. Comparison of the score and loadings plots indicates that the latter separation mainly results from the very high content of ferulic acid (see also **Table 2.3**). The four bran samples do not cluster in the score plots, but pairs of samples group together in all three plots and these pairs can be distinguished by their high and low contents of p-coumaric acid as seen in loading plots.

By contrast, the free + conjugated fraction does not show any clear clustering (**Figure 2.5**). However, a grouping of a flaked and oatbran sample is observed in score plots, which could be explained, by the avenanthramide A and B content of these products seen in the loading plots. Because bound phenolic acids represent about 80% of the total, PCA analysis of the total fraction was essentially similar to that of the bound fraction (not shown).

The greater variation in the contents of free + conjugated components compared to bound, both within and between the types of sample, may reflect wider variation in the composition of the raw material. For example, studies of wheat have shown that the growing conditions have a much greater impact on the content and composition of free and conjugated phenolic acids than on bound phenolic acids, with heritabilities calculated as 6.4%, 9.8% and 28.5%, respectively (304). The solubility of these components could also lead to greater losses during processing.

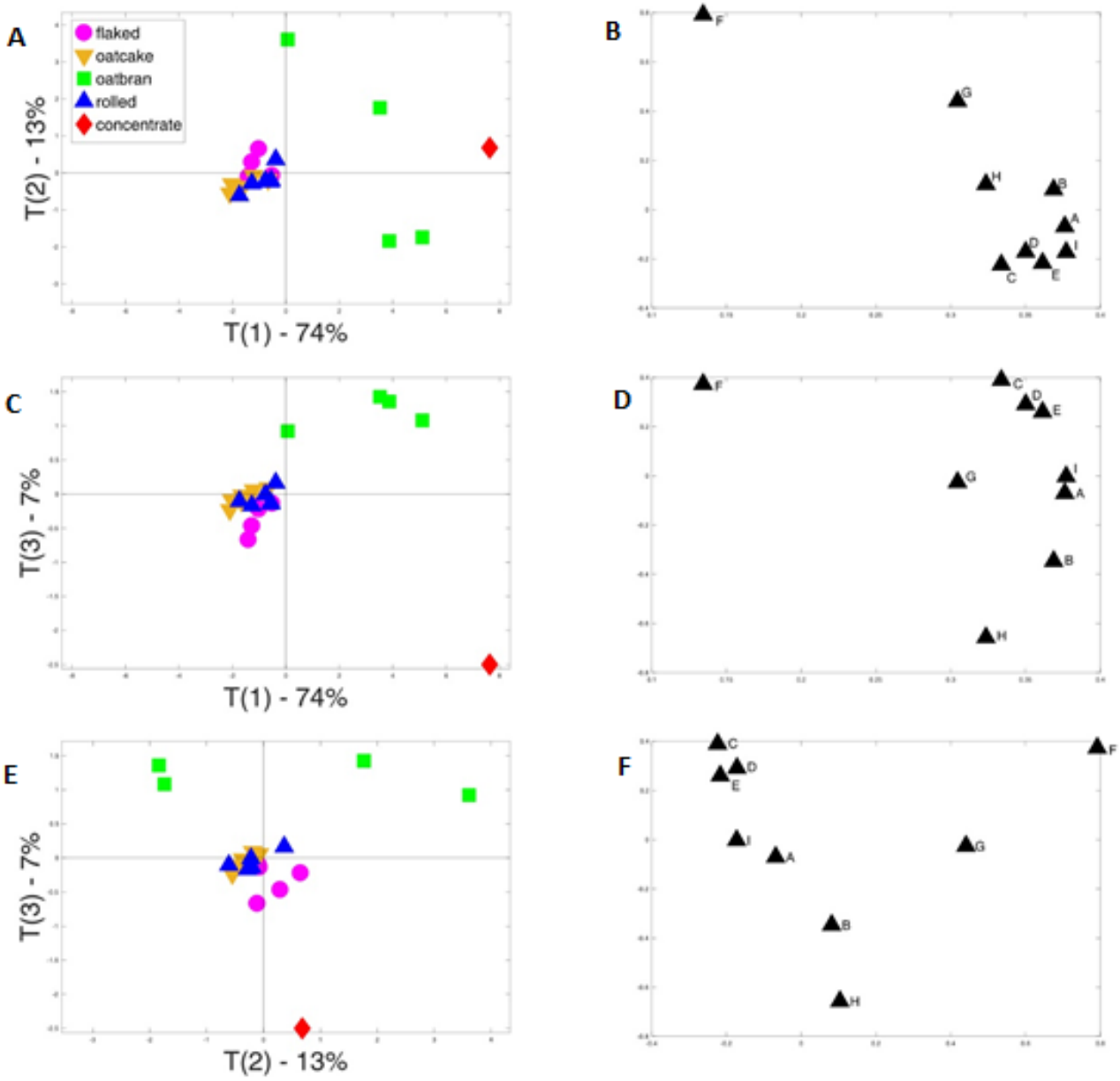

Figure 2.4 Principal component analysis of phenolic acid content of different oat products, bound fraction. A, C, E represents score plots which show the grouping of oat products based on their overall phenolic acid compositions and B, D, F represents corresponding loading plot of each score plot which show the individual phenolic acids responsible for the separation. Letter codes seen in loading plots respresent phenolic acids: A 4-Hydroxybenzoic acid, B Caffeic acid, C Vanillic acid, D 4-Hydroxybenzaldehyde, E Syringic acid, F *p*-Coumaric acid, G Vanillin, H Ferulic acid, I Sinapic acid.

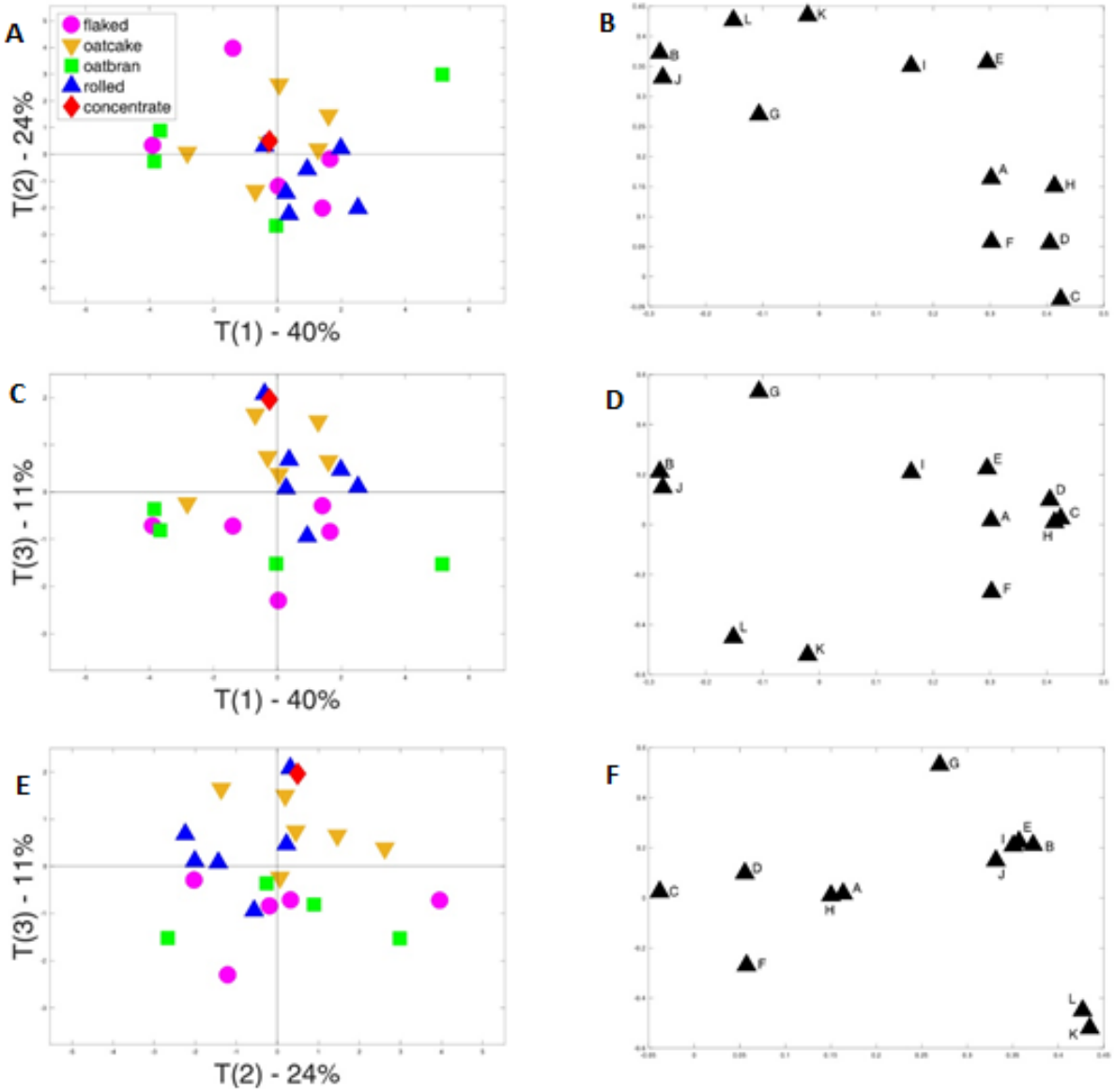

Figure 2.5 Principal component analysis of different oat products, free+conjugated fraction. A, C, E represents score plots which show the grouping of oat products based on their overall phenolic acid compositions and B, D, F represents corresponding loading plot of each score plot which show the individual phenolic acids responsible for the separation.Letter codes seen in loading plots respresent phenolic acids: A 4-Hydroxybenzoic acid, B Caffeic acid, C Vanillic acid, D 4-Hydroxybenzaldehyde, E Syringic acid, F p-Coumaric acid, G Vanillin, H Ferulic acid, I Sinapic acid, J Avenanthramide C, K Avenanthramide A, L Avenanthramide B.

2.4.5 Potential Health Benefits of Phenolic Acids and Ferulic Acid and Intake from Commercial Oat Products

It has been suggested that the consumption of dietary phenolics may reduce the risk of chronic diseases such as CVD, diabetes, cancer and neurodegenerative diseases (305). The average daily intake of phenolic acids in Europe is 605 mg/day, with wholegrains contributing about 5.5% of the total, while the ferulic acid intake is 38 mg/day (306). Previous studies carried out in humans reported significant improvements in vascular function following a single dose of phenolics in 375 ml of champagne (61.63 mg/l) and chlorogenic acids (89 and 310 mg/g) in 3.6 g of coffee (30, 31). Our study showed that the consumption of a 40g portion of commercial oat products provides from 15.8 to 25.1 mg total phenolics (from 9.9 to 19.3 mg bound and from 4.9 to 5.7 mg free+ conjugated phenolics) including 1.1 - 2 mg avenanthramides and 16.7 mg of total phenolics (15.2 mg of bound and 1.5 mg of free + conjugated phenolics) including 1.2 mg of avenanthramides in a 11g portion of oat bran concentrate.

Ferulic acid is the major component present in all commercial oat products and our analyses show that between 9.1 and 14.7 mg of total ferulic acid (from 7.6 to 13.3 mg bound and from 1.4 and 1.6 mg free + conjugated fraction) can be provided in a 40g portion of commercial oat products and 13.1 mg of total ferulic acid (12.7 mg of bound and 1.3 mg free + conjugated fraction) can be provided in a 11g portion of oat bran concentrate. A number of studies have reported therapeutic effects of ferulic acid against diseases related to oxidative stress, with an increasing body of evidence for a role in the prevention or management of chronic disease (307). For example, randomized controlled trials to determine the effect of coffee and cranberries on vascular health showed improvements on outcomes such as flow mediated dilatation (FMD) and these findings were correlated with the presence of ferulic acid metabolites (30, 283). We would therefore predict that the consumption of oat phenolics, and ferulic acid in particular, may have similar health benefits.

In conclusion, this study showed that wholegrain oat products can provide a source of phenolic compounds. The total concentrations of phenolic acids and avenanthramides ranged from 394.8±24.2 μg/g to 1518.6 μg/g, with oat bran concentrate having the highest content and the other wholegrain products and oat bran containing lower but similar amounts. However, the total contents of free + conjugated phenolic acids and avenanthramides were similar in all products. This is important as this fraction should be more bioaccessible and absorbed in the small intestine, whereas the bound fraction will only be released, at least partially, during fermentation in the colon. Consequently, similar potential health benefits could be delivered by all products analysed.

These data could be useful to inform studies of human dietary intake and interventions with oat products to better understand the health benefits of oats.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgements

The authors acknowledge support from the Biotechnology and Biological Sciences Research Council of the UK (Grant BB/M002802/1 Development and application of phenolic-rich oats for the maintenance of cardiovascular health). Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK and the work forms part of the Designing Future Wheat strategic programme (BBS/E/C/000I0250).

Chapter 3 Acute consumption of phenolic acid and avenanthramide rich oats improves microvascular function in pre- and stage 1 hypertensive men and correlates with plasma metabolites

3.1 Abstract

Background : Wholegrain consumption is linked to lower risk of cardiovascular disease. Randomized controlled trials (RCTs) have established that the consumption of wholegrain oats lowers blood cholesterol and improves post-prandial glycaemic control. While some of these benefits are mediated by β-glucan fibre, the extent to which oat phenolic acids and avenanthramides are involved is unknown.

Objective: We examined the potential acute effects of phenolic-rich oat intake on vascular function and assessed potential underlying mechanisms by the assessment of NADPH oxidase activity and circulating metabolites in men with prehypertension and stage 1 hypertension relative to a fibre and macronutrient-matched control.

Design: A 2-arm, randomised, crossover, single-blind clinical trial was conducted with 16 male participants. Volunteers received either 90.2g oats containing 5mg avenanthramides and 45mg phenolic acids or a macro- and micronutrient matched rice control containing 4.2mg phenolic acids and 0 mg avananthramides. Vascular function was measured at baseline and post-treatment (i.e. 1, 2, 4, 6 and 24 h). Serum and urine phenolic acids, avenanthramides and their metabolites were analysed by (UPLC)-MS/MS.

Results: Flow mediated dilation was not significantly different between treatments, although there was a small improvement in FMD at 1h (0.21 ± 0.7%; p =0.5) and 24h (0.28 ± 0.4%, p=0.1), following oat intake relative to the control. Significant increases in both endothelium dependent (iAUC, p=0.04, and AUC p=0.02) and independent (iAUC, p=0.007) microvascular function were observed after consumption of the oat intervention relative to control at 2h. We observed large variations in phenolic acid absorption between participant's, 2h after intake, and changes in microvascular function were positively correlated with the blood concentration of eight phenolic acid metabolites at 2h, notably ferulic acid 4-O-β-D-glucuronide, ferulic acid-4-O sulfate and p-coumaric acid. No significant changes in blood pressure or NADPH oxidase activity were observed between treatments.

Conclusion: This study indicates that consumption of oats delivering 50mg of phenolic acids and avenanthramides acutely improves microvascular function in pre- and stage 1 hypertensive men which appears to be partly mediated by the appearance and concentration of specific phenolic acid metabolites in the circulation.

3.2 Introduction

Epidemiological studies suggest that regular consumption of whole grains is associated with a lower risk of cardiovascular disease (CVD), the meta-analyses of multiple human intervention studies are also providing strong evidence that regular oat (Avena sativa) intake lowers blood cholesterol, improves insulin sensitivity and post-prandial glycaemic control (58, 271, 275, 281). A health claim approved by European Food Safety and Authority (EFSA) states the daily consumption of 3 g of oat soluble fibre , β-glucans can lower blood cholesterol and improves heart health (20, 58, 271). Studies have also suggest oats may reduce blood pressure, but the results are mixed and evidence is not conclusive (25, 308). While oats contain a range of macro and micronutrients such as proteins, unsaturated fatty acids, dietary fibre in the form of arabinoxylan and β-glucan, vitamins and minerals (282), they are also a rich source of phenolic acids, particularly ferulic acid and structurally similar avenanthramides (21). These compounds present in oats in three forms: 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, notably cell wall polysaccharides and lignin(289).

Existing scientific data from epidemiological and human intervention studies suggest that polyphenol-rich foods have a range of beneficial effects on cardiovascular health (23, 309-311). The ability of such foods to attenuate the incidence of CVD risk factors such as endothelial dysfunction, hypertension, abnormal lipid profile and platelet activation in at-risk individuals has been the main focus(23, 311). Their potential to influence endothelial nitric oxide synthase (eNOS) and increase nitric oxide (NO) bioavailability is likely to underpin the actions at the physiological level(187). NO 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 (38). Studies suggest that phenolic acids and avenanthramides may contribute to the beneficial effects of a diet rich in oats and clinical trials have shown protective effects of circulating phenolic acids against CVD (29, 30, 283, 312). For example, an acute intervention with cranberry juice, increased flow mediated dilation (FMD) in healthy men and this finding was correlated in time with changes in plasma cranberry phenolic acids such as ferulic and caffeic acid sulfates (283). Similarly, an acute investigation of blueberry flavonoids in healthy men has shown to increase the FMD response and correlated well with plasma metabolites such as vanillic acid, benzoic acid, hippuric acid, hydroxyhippuric acid and homovanillic acid(29). Additionally, those small phenolic compounds including hippuric, vanillic, and homovanillic acids predicted declines in NAPDH oxidase activity in neutrophils which might also increase NO bioavailability and improve endothelial function(28, 29). Coffee chlorogenic acids have also improved FMD acutely and appeared in the circulation as phenolic acids such as ferulic acid sulfate and isoferulic acid glucuronide which were linked with the vascular

improvement(30). Avenanthramides in oats have also been shown to be bioavailable in humans and may exert anti-inflammatory and vasodilatory effects to protect against CVD (27, 165, 313-315). In order to exert health benefits, free and conjugated phenolic acids and avenanthramides and/or their methylated, sulfated or glucuronidated metabolites are likely to be absorbed early in the small intestine whereas bound phenolic acids might reach to colon where they may be subject to extensive catabolism by colonic microbiota and converted into bioactive phenolic acids and/or metabolites (149, 152, 316).

There is limited evidence with respect to the potential of oat intake on the vascular system and a reduction in CVD risk (277, 278, 280). However, these studies do not differentiating the impact of phenolic acids and avenanthramides from fibre and the contribution of these compounds to health benefits of oats is still not known. Considering the evidence from RCTs examining other phenolic containing foods and stating that small phenolic acids are bioavailable and have the potential to mediate vasodilatory effects, we tested the acute intake of phenolic rich oat based breakfast or a soluble fibre matched cream of rice to investigate potential vascular benefits of oat phenolic acids and avenanthramides in pre-and stage 1 hypertensive men. Vascular function was mainly assessed by FMD and LDI and correlated with serum phenolic acid and avenanthramides and/or their metabolites to provide a cause and effect relationship.

3.3 Materials and Methods

3.3.1 Materials

All phenolic acid aglycones were obtained from Sigma-Aldrich (Gillingham, UK) except 4-hydroxyhippuric acid and 3-hydroxyhippuric acid which were purchased from Enamine (Kiev, Ukraine). All the phenolic acid metabolites (glucuronides and sulfates) were obtained from Toronto Research Chemicals Inc (Toronto, Canada). All solvents were LC MS grade and were obtained from either Sigma-Aldrich or Fisher Scientific (Loughborough, UK).

3.3.2 Study Intervention

The study intervention materials comprised of 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 to make oat based breakfast. 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 3.97 mg phenolic acids and was closely matched to oat porridge for macro- , micronutrient content and soluble fibre content (**Table 3.1**). β- glucan, betaine, choline and trigonelline were present at levels too low to induce any observable vascular effects (317-319).Oat interventions were measured by using HPLC as previously described in Chapter 2 (320) for phenolic acid and avenanthramide content and analysed for macro and micronutrients by Campden BRI (Gloucestershire, UK). All dry materials were frozen until needed to prevent degradation. Both interventions were well tolerated by all subjects, and no adverse events were reported.

Table 3.1 Nutritional analysis of the study intervention materials

[1] 67.7g CDC dancer oat flakes, 22.5g oat bran concentrate and 420ml water containing 5.6g of β -glucan as soluble fibre.
[2] 39.4g cream of rice, 6.1g sunflower oil, 29.5g skimmed milk, 5.6g pectin powder as soluble fibre, 6.5g cellulose and 420ml water.

	Oat intervention [1]	Control intervention [2]
Energy (kcal)	322.0	322.0
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
Protein (g)	13.3	13.3
Total Phenolics (mg)	50.0	3.97
Conjugated and Free Fraction (mg)	11.96	0.89
4-Hydroxybenzoic acid	0.85	0.14
Vanillic acid	0.63	0
4-Hydroxybenzaldehyde	0.25	0
Syringic acid	1.38	0
p-Coumaric acid	0.33	0.11
Vanillin	0.27	0
Ferulic acid	3.40	0.37
Sinapic acid	3.30	0.27
Caffeic acid	1.55	0
Bound Fraction (mg)	33.04	3.08
4-Hydroxybenzoic acid	0.79	0
Vanillic acid	0.53	0.05
4-Hydroxybenzaldehyde	0.21	0
Syringic acid	0.85	0
p-Coumaric acid	0.09	0.19
Vanillin	0.23	0
Ferulic acid	24.93	2.74
Sinapic acid	3.16	0.11
Caffeic acid	2.24	0
Total Phenolic acids (mg)	45.02	3.97
Aveanthramide A	1.31	0
Avenanthramide B	2.93	0
Avenanthramide C	0.77	0
Total Avenanthramides (mg)	5.01	0

3.3.3 Subjects

Pre- and stage 1 hypertensive (SBP 120-159 mmHg and DBP 75-99 mm Hg) male subjects (n 16) aged between 25 and 75 years, were recruited by study researchers from the University of Reading and surrounding area(**Figure 3.1**). Individuals with hypertension (SBP/DBP ≥ 160/100 mm Hg), body mass index (BMI) > 35, abnormal biochemical and haematological results as assessed at health screening, past or existing medical history of vascular disease, diabetes, hepatic, renal, gastrointestinal, haematological, neurological, thyroidal disease or cancer, current smoker or ex-smoker, allergic to whole grains and taking any dietary supplements, lipid lowering, antihypertensive, vasoactive, anti-inflammatory, antibiotic or antidepressant medication, having flu vaccination or antibiotics within 3 months of trial start, having vigorous exercise (more than five times x 30 min per week), or consuming alcohol ≥21 units /week were excluded from the study. Health was ascertained by medical questionnaire and normal concentrations of plasma glucose, total cholesterol, triglyceride, creatinine, bilirubin, uric acid and liver enzymes (aspartate aminotransferase, alanine aminotransferase and g-glutamyl transferase) levels and normal haemoglobin concentration, packed cell volume and leukocyte counts at the screening visit.

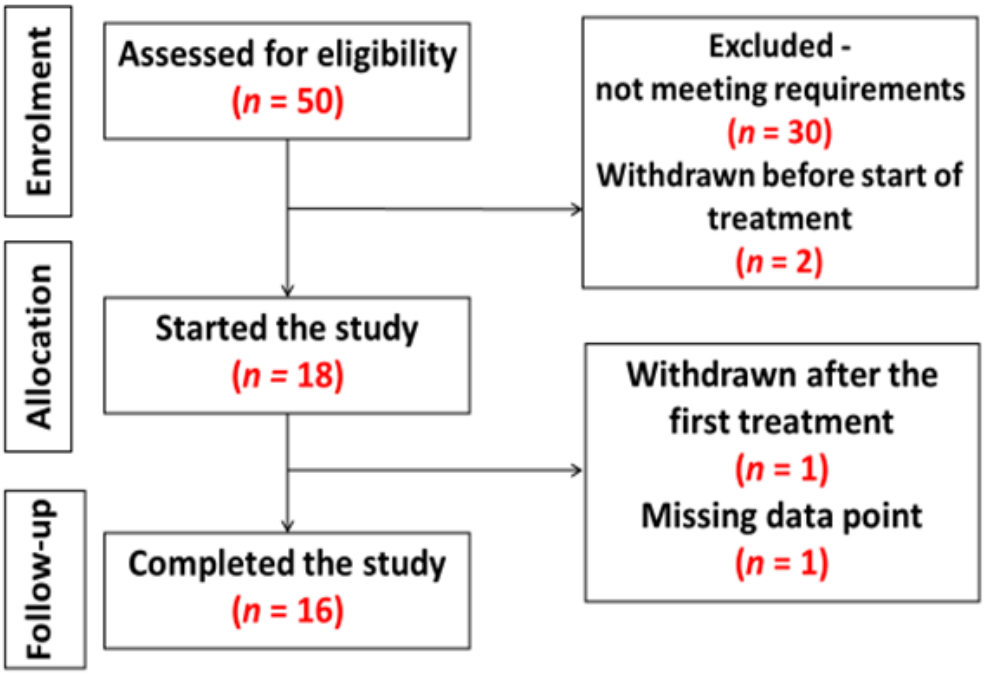

Figure 3.1CONSORT study flow diagram

3.3.4 Study Design

A two-arm, single-blinded (researcher blinded; participant not), placebo-controlled randomised crossover intervention trial was conducted where volunteers were asked to consume an avenanthramide and phenolic acids-rich porridge or an energy and fibre matched control intervention. FMD, blood pressure and blood samples were taken before (baseline) and at 1, 2, 4, 6 and 24h post-acute consumption of each treatment on two different study visit days by 2 weeks washout. LDI and NADPH oxidase activity were measured at 0, 2, 4, 6 and 24h and urine samples were collected at 0, 3, 7 and 24h post consumption (**Figure 3.2**).

FMD of the brachial artery to assess vascular endothelial function was the primary endpoint of the study (detailed below). Secondary endpoints included LDI, blood pressure and measuring NADPH oxidase activity (all detailed below). Tertiary endpoint was quantification of serum and urine oat avenanthramides and phenolic acids to investigate the link between primary and secondary endpoints. Before vascular assessments, body composition and weight were measured, using a Body Composition Analyser (Tanita BC-418MA, TANITA, UK). 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). Office blood pressure was also measured three times after 15 mins of rest in a temperature-controlled (22 ± 1 °C), dimmed room using an automated clinical digital sphygmomanometer (Omron M6 Intelli-Sense Comfort; model HEM-72211-E8, Omron Healthcare Co.) with appropriately sized cuff in the subject's non-dominant arm.

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) and to restrict their diet, which included refraining from consuming polyphenol-rich foods such as oats , oat products, wholegrains, fruits, vegetables, spices, chocolate, nuts, seeds, coffee, tea, plant-based milk, caffeinated drinks, wine, beer and spirits, moderate the consumption of alcohol to ≤ 21 units /week and to refrain in exercise 48 h prior to the study visits. Compliance to the diet and lifestyle restrictions was verified with 24h-dietary recall sheet.

After completion of baseline measurements, an independent researcher who previously generated computer based paper list of random number sequences and volunteer allocation also provided the intervention products, which the volunteers were asked to consume completely over a 15min period. To minimise bias study researchers were 'blinded' to the food (intervention) products. 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 water ad libitum, while otherwise consuming just the meals as provided. 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.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the University of Reading Research Ethics Committee (ref. 15/60). The study was registered with the National Institutes of Health (NIH) randomised trial records held on the NIH ClinicalTrials.gov website (ref.NCT02731755) Written informed consent was obtained from all subjects before the study started.

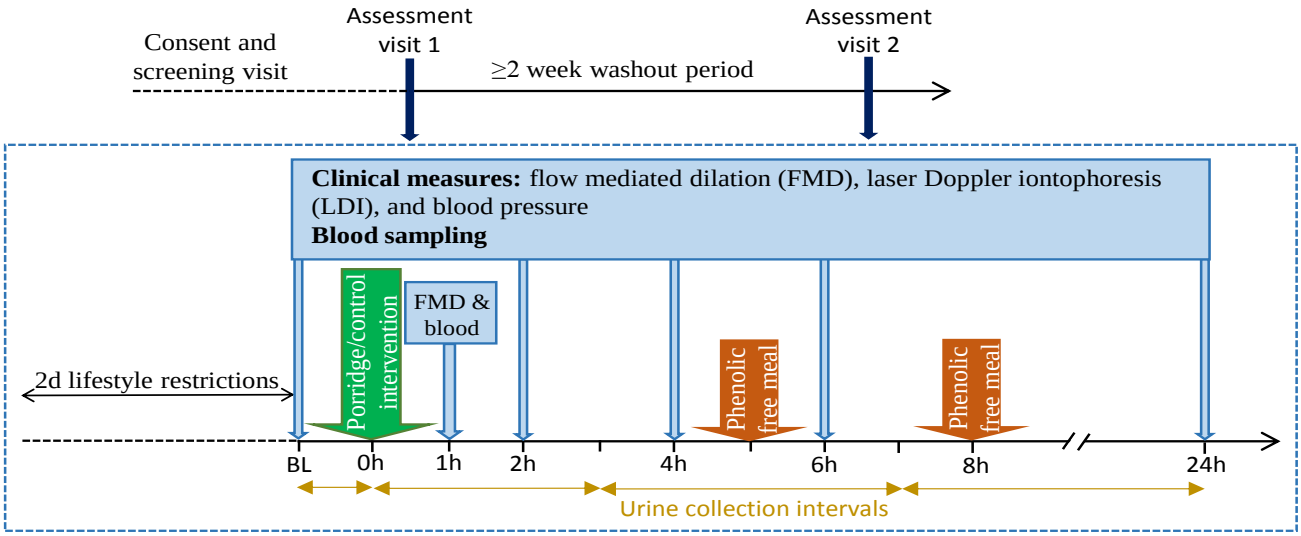

Figure 3.2 Study design

3.3.5 Plasma- urine collection and biochemical analysis

Blood samples were collected in lithium-heparin and serum separating tubes (Greiner Bio-one, USA). A qualified research nurse was involved in the study to cannulate the volunteers for the blood collection. Thereafter, the lithium-heparin tubes were used immediately for NADPH oxidase activity analysis whereas serum separating tubes were allowed to coagulate for 30-60 minutes before centrifugation for 15 min at 1700 X g at 4 ˚C, in order to separate the serum. Collected urine was kept on ice packs, the excretion volume measured and was spun for 15 min at 1700 X g at 4 ˚C. All samples were frozen at-80˚C until analysis.

All biochemical parameters to determine baseline characteristics of volunteers were assayed by ILAB 600 chemistry analyser (Instrumentation Laboratory, Warrington, Cheshire, UK) using enzyme based colorimetric tests supplied by Instrumentation Laboratory. The following parameters were determined in all samples: total cholesterol, triglycerides and glucose.

3.3.6 Flow-Mediated Dilatation (FMD)

Endothelial-dependent vasodilation was measured according to standard guidelines by using an ATL Ultrasound HDI5000 system (ATL Ultrasound) together with a semiautomated computerized analysis system (Brachial Analyzer; Medical Imaging Applications-llc)(321). The procedure used an ECG-gated trigger and the image-grabbing software (MIA-llc) to collect images at 0.25 frames/s. 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. 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. After 5 min of occlusion, the pressure was rapidly released, allowing reactive hyperaemia to occur; measurement collection continued for 5 min post release. Image files were analysed by a single researcher who was blinded to the measurement details, and peak diameter was 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.

3.3.7 Laser Doppler Iontophoresis (LDI)

LDI is a validated technique used as a surrogate marker of microvascular function (322, 323). It is non-invasive and quantifies vasodilator responses in the forearm after stimulation with 1 % acetylcholine (Ach) (endothelium dependent vasodilatation) and 1 % sodium nitroprusside (SNP) (endothelial-independent vasodilatation), delivered transdermally using iontophoresis. In all subjects, measurements were carried out in a temperature-controlled room (22 ± 1 °C) 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 acetylcholine chloride (2·5 ml; 1 % (w/v) in 0·5 % (w/v) NaCl solution; Sigma Aldrich, Poole, Dorset, UK) and sodium nitroprusside (2·5 ml; 1 % (w/v) in 0·5 % (w/v) NaCl solution; Sigma Aldrich).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, Axminster, Devon, UK). Scans with 5 µA incremental increases in current from 0 to 20 µA and skin perfusion, or erythrocyte flux were measured to determine the elasticity of the small peripheral blood vessels at the lower arm. 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 (324).

3.3.8 NADPH Oxidase Activity

Inhibitory action of oat phenolic acids on neutrophil NADPH oxidase activity in whole blood samples was measured with a flow cytometry assay modified from previous studies (29, 325). Whole blood aliquots (50µL) were incubated in the presence of phorbol myristate aceate (PMA) (50 ng/mL) (Sigma) for 45 min at 37°C to stimulate superoxide production by neutrophils. The un-stimulated and blank tubes were also incubated likewise, but in the presence of phosphate buffered saline (Sigma) instead of PMA. Dihydrorhodamine 123 (30µg/mL) (Sigma) was added together with antibody CD16 (20µl) (Becton Dickson) and incubated for 5 min at 37°C. Red blood cells were lysed with 1 mL of fluorescent activated cell sorting lysing solution (Becton Dickson) and incubated for 20 min in the dark at room temperature. The cells were then centrifuged at 400 x *g* for 5 min at 4 °C. 900µL of supernatants were discarded from the centrifuged tubes and cells resuspended with 1 mL of 2% fetal calf serum (Sigma) and 0.2% ethylenediaminetetraacetic acid (EDTA) in PBS. The tubes were then centrifuged again at 400 x *g* for 5 min at 4°C. A final 950µL of supernatants were discarded from the tubes, and 400µL of paraformaldehyde (1%) (Sigma) added to fix the cells. Data were analysed by the Accuri C6 flow cytometer (Becton Dickson), and median intensity of fluorescence was used to

evaluate the fluorescence of each sample tube. The NADPH oxidase activity was calculated as a percentage difference between values obtained in PMA activated and resting cells.

3.3.9 Solid Phase Extraction

Phenolic acids were extracted from serum and urine using a validated method as previously described (See Appendix) (152, 326). Briefly, 1 mL of urine or serum was spiked with an internal standard (i.e. 3,5-dichloro-4-hydroxybenzoic acid) and subsequently extracted using SPE cartridges (Strata-X columns 500 mg $6mL^{-1}$; Phenomenex). Cartridges were washed with 12 mL of 0.1/99.9 v/v hydrochloric acid/water, dried for 30 min under vacuum, soaked in 0.1/99.9 v/v hydrochloric acid/methanol for 10 min, and eluted into glass vials with 7 mL 0.1/99.9 v/v hydrochloric acid/methanol. Samples were evaporated to complete dryness under speedvac centrifugal evaporator (Savant™ SPD131DDA SpeedVac™ Concentrator, ThermoFisher Scientific) at room temperature. The dried samples were resuspended in 250µL of mobile phase (0.1/5/94.9, v/v/v, formic acid/methanol/water) by 30 s vortexing, 15min ultrasound sonicating, and 1 h shaking. Serum samples were filtered with using Acrodisc syringe filter with 13 mm, 0.45µm pore size (Sigma-Aldrich). Samples were stored at −80 °C until analysis.

3.3.10 UPLC-MS/MS Analysis

Serum and urinary analysis of phenolic acid metabolites were performed with UPLC–ESI–MS/MS system consisted of an Aquity UPLC Hclass (Waters) coupled to a Xevo TQ-S micro ESI mass spectrometer (Waters) operated using MassLynx software (V4.1, Waters Inc, USA) as previously described, using a validated method (152). Briefly, compound separation was achieved using an Aquity UPLC HSS T3 1.8µm column (2.1 × 100 mm) attached to a Van guard precolumn of the same material and pore size, maintained at 45 °C with a flow of 0.65 mL min^{-1} and a sample injection volume of 2 µL. The mobile phase consisted of 0.1/99.9 v/v formic acid/water (A) and 0.1/99.9 v/v formic acid/acetonitrile (B); and a mobile phase gradient consisting of: 1% B at 0 min, 1% B at 1 min, 30 % B at 10 min, 95 % B at 12 min, 95% B at 13 min, 1% B at 13.10 min, 1% B at 16 min. The scheduled multiple reaction monitoring (sMRM) method which was previously developed by syringe infusion of 34 analytical standards was further optimized with the addition of commercially available phase II metabolites and phenolic acids to determine sMRM transitions, optimal sMRM modes (i.e. negative or positive) and collision energies(**Table 3.2**).

Regarding to compounds without analytical standards (i.e benzoic acid-*O*-sulfate, vanillin/4-hydroxyphenyl acetic acid-*O*-sulfate, hippuric acid-*O*-sulfate, caffeic acid-*O*-sulfate, syringaldehyde-*O*-sulfate, syringic acid-*O*-sulfate, sinapic acid-*O*-sulfate, dihydro avenanthramide C and dihydro avenanthramide B), retention times and sMRM transitions were tentatively identified by injecting a pooled extract of urine and serum (i.e. using samples collected after oat intervention intake from all participants and during all post-intake time intervals, *n* = 14 and n=13, respectively). sMRM transitions were taken from the literature(152, 313). Collision energies were optimized to a limited extent by injecting the pooled extract three times and the best one was selected for each sMRM transition. In the final sMRM, a total of 50 potential compounds were targeted. Identification of the phenolic acids for which standards were available was based on their retention times and the major sMRM ion transitions of the standards, while the tentative identification of phenolic metabolites for which analytical standards were not available was based on more than one sMRM ion transitions and the strongest one was selected for quantification (**Table 3.2**).

Quantification was established using the most intense sMRM transition and calibration curves of analytical standards. Where pure standards were not available, quantification was conducted relative to standard curves of compounds with similar structures (e.g. the calibration curve of isoferulic acid-3-*O*-sulfate was used to quantify all tentatively identified sulfate metabolites). The LOD and LOQ were established for each compound as the concentrations of peaks with S/N of 3 and 10, respectively. Blanks and quality controls were run every ten injections.

Table 3.2 LC-MS/MS identification of phenolics and metabolites in serum and urine after the consumption of oat intervention[1]

All compounds were analysed at negative ion mode except vanillic acid, isovanillic acid and 4-methoxy benzoic acid.

Compounds	RT (min)	MW (g/mol)	sMRM ion transitions (m/z)	CE (V)	LOD (nM)	LOQ (nM)	R^2
3,4-dihydroxybenzoic acid-3-O-sulfate	3.33	232.2	233.2/153	13	0.91	3.03	0.999968
3,5-dihydroxybenzoic acid 3-O-sulfate	2.75	232.2	233.2/153	15	0.93	3.10	0.999925
4-methoxybenzoic acid	8.52	152.15	153.3/109	13	1021.90	3406.33	0.999829
Caffeic acid 3-B-D-glucuronide	4.93	356.28	355.2/179	20	2.17	7.23	0.999991
Caffeic acid 4-B-D-glucuronide	4.93	356.28	355.2/179	17	0.41	1.38	0.998562
Dihydro caffeic acid 3-O-sulfate	4.95	260.25	261.24/181	15	30.16	100.52	0.999664
Ferulic acid-4-0-sulfate	6.06	274,243	273.25/193	13	600.58	2001.92	0.999731
Ferulic acid-4-B-D-G-glucuronide	5.5	370.31	369.27/193	14	2.26	7.54	0.999731
Isoferulic acid-3-O-sulfate	6.57	272.25	273.25/193	15	397.46	1324.87	0.999702
Dihydro isoferulic acid 3-O-B-D-glucuronide	6.52	372.32	371.3/113	16	61.33	204.44	0.999789
Dihydroferulic acid 4-O-sulfate	5.81	276,263	275.27/195	15	0.27	0.90	0.999558
Homovanillic acid sulfate	4.95	262,232	261.24/181	13	3.41	11.36	0.999930
p-coumaric acid 4-O-B-D-glucuronide	4.95	338.27	339.28/113	13	0.27	0.91	0.998376
p-coumaric acid 4-O-sulfate	5.68	244.22	243.24/163	11	0.20	0.65	0.999806
p-salicyclic acid-4-sulfate	3.72	216.2	217.2/137	16	1.18	3.95	0.999946
Salicylic acid-B-D-O-glucuronide	5.28	314.24	313.1/175	8	25.88	86.26	0.999991
Dihydro avenanthramide-B	10.31	331	330/386.1	15	0.11	0.38	NA
Dihydro avenanthramide-C	14.14	317	316/272.02	10	0.12	0.40	NA
4-hydroxybenzoic acid	4.85	138,122	137.2/93	13	119.52	398.40	0.999969
Vanillin	6.19	152.15	151.15/136	12	130.34	434.45	0.999324
4-hydroxyphenylacetic acid	4.77	152.15	151.15/107	10	104.86	349.55	0.999910
2,4-dihydroxy benzoic acid	4.75	154.12	153/109	11	191.93	639.77	0.999946
3,5-dihydroxy benzoic acid	3.72	154.12	153/109	13	291.62	972.06	0.999786
2,5-dihydroxy benzoic acid	4.76	154.12	153/109	11	282.60	942.00	0.999946
p-Coumaric acid	6.95	164.05	163/119	13	231.72	772.40	0.999993
Isovanillic acid	6.11	168	169/93	12	962.11	3207.04	0.999704
Vanillic acid	5.8	168.14	169/93	12	1962.22	6540.73	0.999865
Hippuric acid	5.53	179.17	178.17/134	11	52.09	173.62	0.998406
Caffeic acid	5.87	180.16	179.2/135	15	314.54	1048.48	0.999746
Homovanillic acid	5.86	182.17	181.17/137	10	153.65	512.17	0.993707
Syringaldehyde	7.35	182.17	181.2/151	18	0.06	0.19	0.999816
Isoferulic acid	7.95	194.18	193.2/178	10	0.16	0.55	0.999150
Ferulic acid	7.64	194.18	193/178	10	0.15	0.50	0.999491
4-hydroxyhippuric acid	4.34	195.17	194/100	11	2.22	7.38	0.999698
3-hydroxyhippuric acid	4.6	195.17	194/100	11	50.45	168.18	0.999979
2-hydroxyhippuric acid	6.8	195.17	194/100	11	61.36	204.54	0.999997
Hydroferulic acid	7.26	196.21	195/136	15	0.29	0.95	0.999703
Syringic acid	6.36	198.17	197/182	13	0.20	0.68	0.998364
Sinapic acid	7.86	224.21	223.2/208	12	0.14	0.48	0.999562
Avenanthramide A	9.86	299.28	298/254	17	0.48	1.60	0.999833
Avenanthramide C	8.99	315.28	314.3/178	16	18.73	62.45	0.999871
Avenanthramide B	10.32	329.31	328.3/268	10	0.05	0.16	0.998983
Dihydroferulic acid-glucuronide	5.88	372.33	371/113	17	179.71	599.03	0.999464
Benzoic acid-O-sulfate	3.65	216	217/137**/173*	11	226.19	753.98	NA
4-hydroxyphenylacetic acid-O-sulfate	6.86	231	231/151**/136*	17	237.44	791.47	NA
Hippuric acid-O-sulfate	4.24	259	258/178**/134*	17	45.11	150.37	NA
Caffeic acid-O-sulfate	5.68	258.2	259/179**/135*	17	71.26	237.54	NA
Syringaldehyde-O-sulfate	4.6	262	261/166**/181*	23	0.29	0.98	NA
Syringic acid-O-sulfate	4.6	278	277/197**/182*	11	0.09	0.30	NA
Sinapic acid-O-sulfate	5.96	304	303/233**/208*	11	63.01	210.05	NA

[1]Abbreviations: RT, retention time; MW, molecular weight; sMRM, scheduled multiple reaction monitoring; m/z, signal to noise ratio, CE, collision energy; LOD, limit of detection (signal/noise=3); LOQ, limit of quantification (signal/noise=10), R^2, linear regression coefficient of standard curve; NA, no analytical standard was available to make a standard curve.** ion transitions for higher intensities which are chosen for quantification of metabolites without standards. *ion transitions with lower intensities.

3.3.11 Sample Size and Statistical Analysis

The power calculation was performed for the primary endpoint (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 study endpoints (i.e. markers of CVD risk and phenolic acid and avenanthramide levels in biological samples) between assessment time points and interventions, a linear mixed model was used to analyse with baseline values as a covariate and time, intervention and interaction between time and intervention as fixed effects. When model showed a significant difference, pairwise comparisons between interventions and time points was performed using *post hoc* analysis with Bonferroni adjustment. Significance was defined as $p < 0.05$, with p-values represented in the figures as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Correlation analysis between increased levels of phenolic metabolites in biological samples and beneficially affected markers of CVD risk was performed using Spearman's correlation coefficient. All statistical analyses were carried out by using SAS version 9.4 software package (SAS institute) and GraphPad Prism version 7 software (GraphPad Software Inc.)

3.4 Results

3.4.1 Baseline characteristics

The baseline characteristics of pre and stage 1 hypertensive male subjects were largely within normal limits, with only BMI being slightly higher than the normal range (n =16) (**Table 3.3**). All interventions were well tolerated by all participants and no adverse events were reported.

Table 3.3 Baseline characteristics of the study participants at screening.

Characteristic	Mean ± SEM
Age (y)	47. ± 4.0
BMI (kg/m^2)	25.5 ± 1.1
Systolic blood pressure (mm Hg)	133.9 ± 2.2
Diastolic blood pressure (mm Hg)	81.1 ± 1.0
Heart rate (bpm)	70.8 ± 3.3
Plasma glucose (mmol/L)	5.4 ± 0.2
Plasma total cholesterol (mmol/L)	5.0 ± 0.3
Plasma triglycerides (mmol/L)	1.5 ± 0.3

All values are means ± SEM

3.4.2 Vascular and microvascular function

There was no significant changes in endothelium-dependent brachial artery vasodilation, measured by FMD following consumption of the oat or control interventions, although a small increase towards an improvement in the FMD response at 1h (0.21 %; p =0.5) and 24h (0.28 %; p = 0.1) following oat consumption relative to the control (**Table 3.4 and Figure 3.3**). In contrast, endothelium-dependent microvascular function, assessed by LDI, significantly increased at 2h following oat intake (AUC: 1678.3 PU/h^{-1}; iAUC: 681.8 PU/h^{-1}) relative to control (1358.3 PU/h^{-1}; iAUC: 463.7 PU/h^{-1}) (**Figure 3.4 A**; AUC: p = 0.02; iAUC: p= 0.04). Similarly, endothelium-independent microvascular reactivity significantly increased at 2h following oat intake (709.43 PU/h^{-1}) relative to control (iAUC: 502.88 PU/h^{-1}) (**Figure 3.4 B**; p=0.007). No other vascular variables (peripheral SBP, DBP, pulse pressure (PP) or heart rate (HR)) were significantly altered following oat intervention relative to control (**Table 3.4**), despite the presence of significant effects of time (P = 0.006, P=0.02 and P < .0001 respectively). The reduction in NADPH oxidase activity, measured at 2h post intervention showed greater reduction following oat intake, although this failed to achieve significance (P=0.7) (**Table 3.4**).

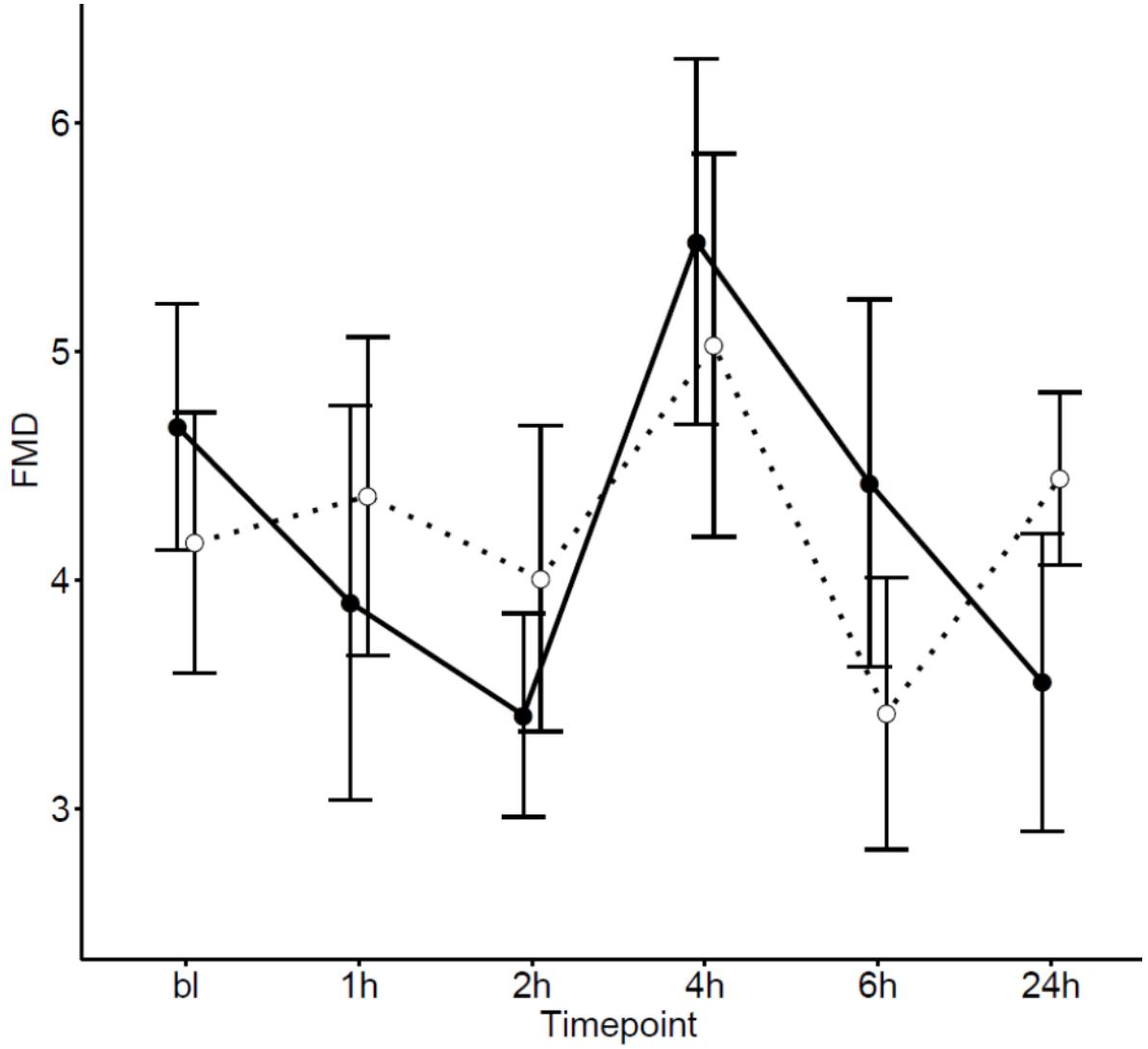

Figure 3.3 Changes in % FMD after consumption of oats and control.

Oats (45.0 mg of phenolic acids and 5.0 mg avenanthramides) (dotted lines) and control (black lines). Values are expressed as means ± SEMs, n=14 from **Table 3.4** at baseline (BL) and 1, 2, 4, 6 and 24 h post intake. Data were analysed using linear mixed model of time, treatment as two factors and followed by Bonferroni *post hoc* test ($P > 0.05$) where there was a significant interaction of time and treatment.

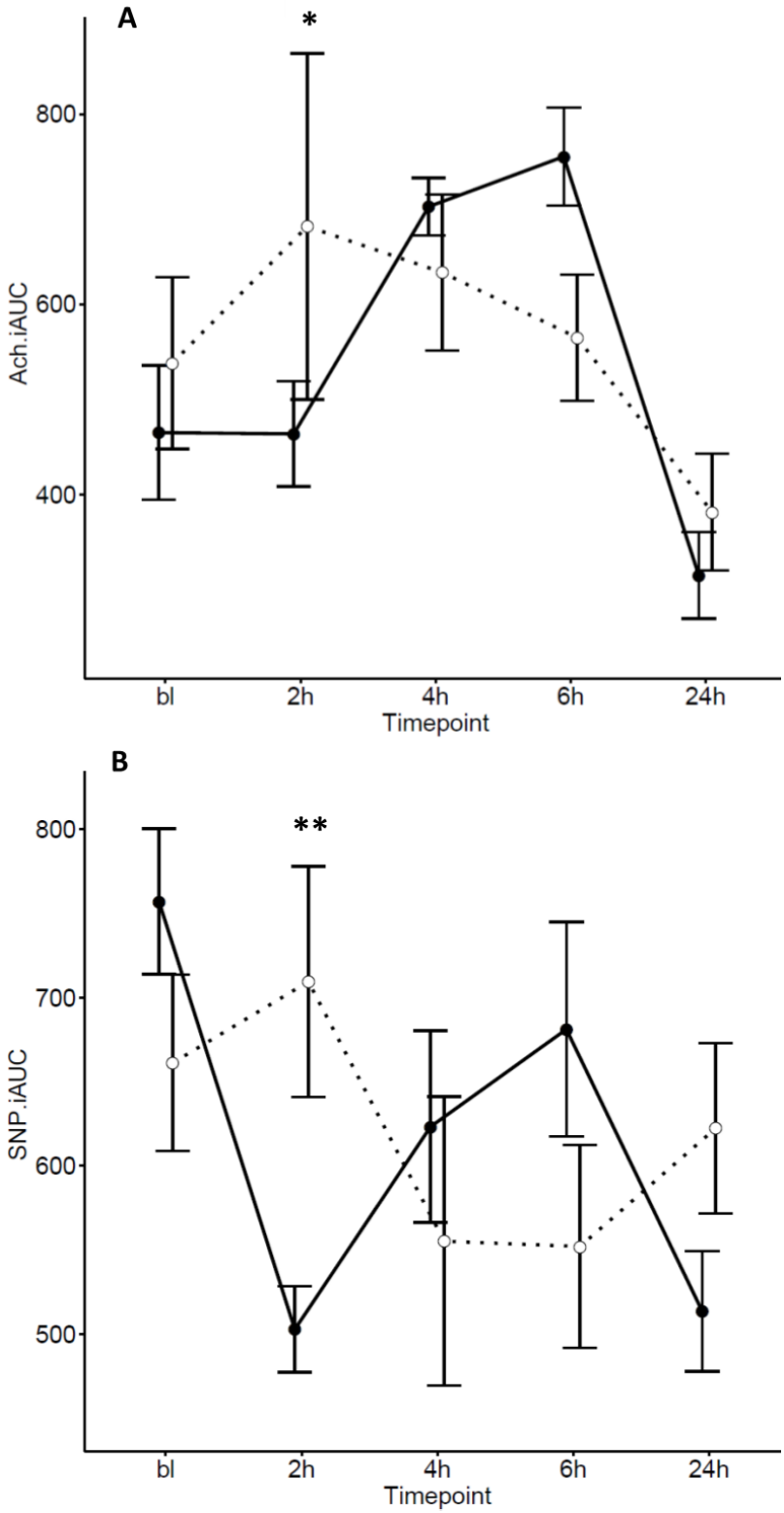

Figure 3.4 Changes in microvascular function measured with LDI after consumption of oats and control

Oats (45.0 mg of phenolic acids and 5.0 mg avenanthramides) (dotted line) and control (black line) : (**A**) endothelium-dependent vasodilation (Ach) represented by incremental area under the curve (Ach iAUC) (perfusion units per hour, PU/h-1) and (**B**) endothelium-independent vasodilation (SNP), represented by incremental area under the curve (SNP iAUC) (perfusion units per hour, PU/h-1).Values are expressed as means ± SEMs from **Table 3.4** at baseline (BL) and 2, 4, 6 and 24 h post intake. A linear mixed model analysis with time and treatment as two factors was performed, n = 13, followed by Bonferroni post hoc test where there was a significance of interaction of both factors. Significant differences compared to control at specified time points are highlighted - *p<0.05 ** p<0.01 and ***p<0.001.

Table 3.4 Measurements of Cardiovascular Risk Biomarkers.

Vascular measures, blood pressure and NADPH oxidase activity of participants at baseline and following the intake of oat and control interventions [a].

Measure	Figure	Intervention A - oats B – control	Baseline	Time after intervention (Hours) 1	2	4	6	24	p-values Treat-ment	Time	Treat-ment × Time
FMD (%)	3.3	A	4.2 ± 0.6	4.4 ± 0.7	4.0 ± 0.7	5.0 ± 0.8	3.4 ± 0.6	4.4 ± 0.4	0.7	0.2	0.5
		B	4.7 ± 0.5	3.9 ± 0.9	3.4 ± 0.5	5.5 ± 0.8	4.4 ± 0.8	3.6 ± 0.7			
Ach-iAUC	3.4 (A)	A	538.2 ± 90.4	-	681.8 ± 182.0	633.4 ± 82.0	564.9 ± 66.3	381.3 ± 61.4	0.9	*** <.0001	* 0.04
		B	465.0 ± 70.6	-	463.7 ± 55.4	702.6 ± 30.4	649.7 ± 51.5	315.0 ± 45.5			
SNP-iAUC	3.4 (B)	A	661.1 ± 52.4	-	709.4 ± 68.4	555.2 ± 85.8	552.1 ± 60.2	622.4 ± 50.6	0.1	0.7	** 0.0074
		B	757.1 ± 43.2	-	502.9 ± 25.6	623.3 ± 57.0	681.1 ± 63.7	513.7 ± 35.8			
Ach-AUC	NS	A	1593.9 ± 198.9	-	1678.3 ± 140.6	1676.4 ± 234.0	1627.1 ± 182.3	1560.9 ± 161.3	0.9	0.3	* 0.0277
		B	1534.1 ± 144.2	-	1358.3 ± 116.9	1621.8 ± 167.5	1757.7 ± 140.8	1701.6 ± 190.9			
SNP-AUC	NS	A	1897.9 ± 159.4	-	1693.6 ± 131.1	1406.2 ± 146.7	1796.0 ± 184.8	1889.9 ± 221.8	0.9	0.1	0.2
		B	2163.4 ± 209.1	-	1694.8 ± 144.7	1723.9 ± 140.1	1760.1 ± 220.5	1903.8 ± 158.3			
SBP	NS	A	125.4 ± 2.3	125.8 ± 2.2	125.7 ± 2.1	124.4 ± 2.3	126.4 ± 1.6	122.4 ± 1.5	0.9	*** 0.0064	0.6
		B	124.4 ± 2.3	124.9 ± 1.8	123.9 ± 1.8	122.3 ± 1.3	127.1 ± 1.9	123.8 ± 1.8			
DBP	NS	A	73.9 ± 1.5	72.8 ± 1.5	73.3 ± 1.5	74 ± 1.7	71.4 ± 1.5	72.5 ± 1.7	0.7	* 0.0213	0.9
		B	74.2 ± 1. 3	72.6 ± 1.3	73.5 ± 1.4	74.6 ± 1.6	72.8 ± 1.4	73.6 ± 1.9			
HR	NS	A	64.7 ± 2.8	63.9 ± 2.5	62.1 ± 2.4	61.3 ± 2.1	62.7 ± 1.7	64.2 ± 2.6	0.9	*** <.0001	0.9
		B	64.9 ± 3.2	63.7 ± 2.7	61.8 ± 2.2	61.1 ± 2.1	65.4± 2.3	64.9 ± 2.6			
NADPH oxidase activity (%)	NS	A	55.7±7.0	-	43.6±5.0	59.8±7.0	56.6±7.0	63.6±6.0	0.5	** 0.0118	0.7
		B	58.6±8.0	-	47.9±8.0	57.5±9.0	51.5±9.0	61.6±9.0			

Flow-mediated dilatation (FMD) (n=14);endothelial-dependent vasodilation(Ach) (n=13) and endothelial-independent vasodilation (SNP) (n=13), expressed as both area under curve (AUC) and incremental area under curve (iAUC); systolic blood pressure (n=14) (SBP); diastolic blood pressure (n=14) (DBP); heart rate (HR);NADPH oxidase activity (n=13). 2 volunteers were excluded from all analyses as the values for vascular measurements were not physiologically relevant for some outcomes or volunteers were found to be not fasted. [a] Values are presented as mean measurement ± SEM, for units see the text. Data were analysed with linear mixed model of treatment, time, and time × treatment interaction, - *p<0.05 , **p<0.01 and *** p<0.001. Post hoc analyses were conducted as Bonferroni's comparison test). NS;not shown, -; measurement is not taken at the timepoint.

3.4.3 Serum and urinary phenolic metabolites

A total of 45 phenolic acids, avenanthramides and their metabolites were identified and quantified in serum (**Table 3.5**). Examples of main phenolic acids and avenanthramides found in oats or their metabolites mainly increasing at 2h following to consumption of oats relative to control are shown in **Figure 3.5 B, 3.5 C and 3.5 D.** Major metabolites were hippuric acid, 4-hydroxyphenylacetic acid, syringaldehyde, isoferulic acid , benzoic acid-O-sulfate, 4-hydroxy benzoic acid and p-coumaric acid glucuronide contributing 10%, 6.9%, 6.3%, 5.7%, 5.6%, 4.5% and 3.2% respectively to the total serum concentration (**Figure 3.5 A and Table 3.5**). An increase in total phenolic acid and avenanthramide metabolites 2h following intake of oat, and to a lesser extent the control was observed (**Figure 3.5 A).** However, although the levels of phenolic metabolites peaked at 2h post consumption of oats, the majority of these increases failed to achieve significance due to a large variation in blood levels between study participants. 3,5-dihydroxybenzoic acid-O-sulfate and p-coumaric acid were the only metabolites to exhibit significant increases following oat intake relative to control (time and intervention interaction: p=0.0008 and p=0.02, respectively) (**Table 3.5**). Urinary analysis showed the presence of total 47 phenolic acids and avenanthramides (**Table 3.6**). Examples of main phenolic acids and avenanthramides present in oats or their metabolites increased following to the consumption of oats relative to control are shown in **Figure 3.6 B, 3.6 C and 3.6 D**. Among 47 compounds quantified in the urine, ferulic acid-4-*O*-sulfate(p=0.03), 2,4 dihydroxy benzoic acid (p=0.02), 2,5 dihydroxy benzoic acid (p=0.01), isovanillic acid (p= 0.02), hippuric acid (p=0.01) , 2 hydroxy hippuric acid (p=0.01), 3 hydroxy hippuric acid (p=0.05), avenanthramide A (p=0.01) and sinapic acid-O- sulfate (p=0.02) increased significantly relative to the control (**Table 3.6**). Predominant metabolites in urine were hippuric acid, benzoic acid-O-sulfate, syringaldehyde-*O*-sulfate, ferulic acid-4-*O*-sulfate, 4-hydroxyhippuric acid and p-salicyclic acid-4-sulfate contributing 28.2%, 10.9%, 9.2%, 8.93%, 6.27% and 5.6% at 3h and 27.3%, 11.7%, 8.6%, 7.7%, 6% and 6.2%, at 24 h respectively, to the total phenolic concentration (**Table 3.6**). Total concentration of phenolic compounds quantified in urine after the consumption oats elevated at 3h (9.72 ±1.3 mg oats vs 7.72 ±1.1 mg control) and also slightly increased at 24h (5.13± 0.71 mg oats vs 4.12± 0.57 mg control) (**Figure 3.6 A and Table 3.6**), although these increases did not achieve significance relative to control intake. Excretion of some of the individual phenolics such as ferulic acid, ferulic-4-*O*-sulfate acid , ferulic acid glucuronide and avenanthramide- A also peaked at 7h as well as 3h after the consumption of oats (**Figure 3.6 B and 3.6 C, Table 3.6**). Phenolics had a urinary recovery of 5.7% after intake of 50 mg of phenolic acids and avenanthramides.

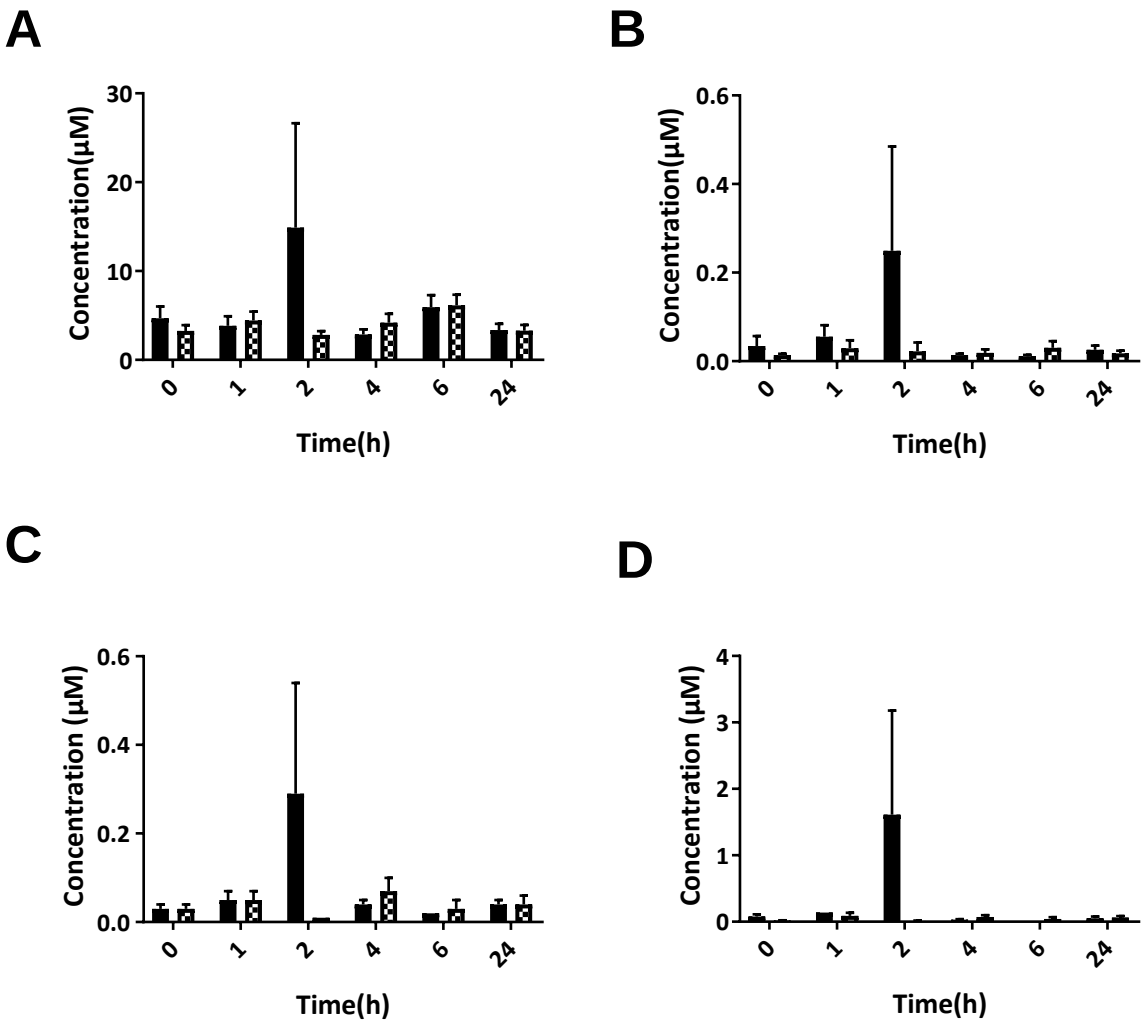

Figure 3.5 Serum concentrations of phenolic acids, aveanthramides and their metabolites following the intake of oats and control.

Serum concentration(µM) measured with UPLC-MS/MS, total (**A**) and main phenolics (**B**; ferulic acid, **C**; ferulic acid glucuronide and **D** avenanthramide B) quantified after the consumption oats (filled bars) and control (dotted bars).Values are expressed as means ± SEMs (n=13). 3 volunteers were excluded from analyses as the values for vascular measurements were not physiologically relevant for some outcomes or volunteers were found to be not fasted. Significance was tested with using a linear mixed model with time and treatment as two factors and when the interaction was significant, post hoc analyses with Bonferroni corrections were performed.

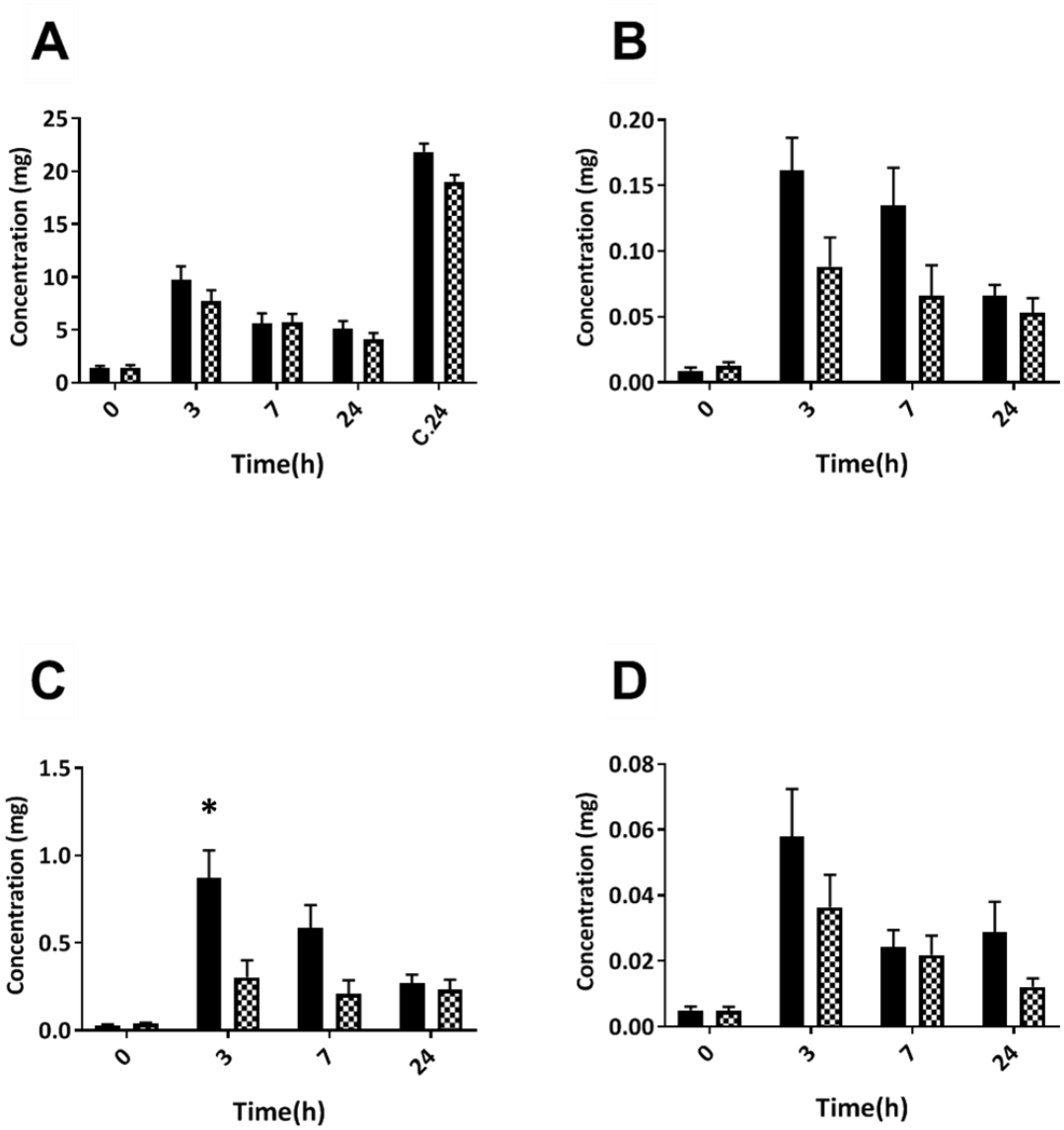

Figure 3.6 Urine concentrations of phenolic acids, aveanthramides and their metabolites following the intake of oats and control.

Hourly urinary excretion of urinary metabolites (mg) measured with UPLC-MS/MS, total (**A**) and main phenolics (**B**; ferulic acid glucuronide, **C**; ferulic acid sulfate and **D** isoferulic acid) quantified after the consumption oats (filled bars) and control (dotted bars).Values are expressed as means ± SEMs (n=13). 3 volunteers were excluded from analyses as the values for vascular measurements were not physiologically relevant for some outcomes or volunteers were found to be not fasted. C.24= cumulative excretion in 24 hours. Significance was tested with using a linear mixed model with time and treatment as two factors and when the interaction was significant , post hoc analyses with Bonferroni corrections were performed.

Table 3.5 Concentrations of individual compounds in serum following the intake of oats and control. **Phenolic acids, avenanthramides and their metabolites detected in serum(nM) at specified timepoints. Values are expressed as means ± SEMs. Significance was tested with using a linear mixed model with Bonferroni corrections (n=13).**

		Phenolic acid concentration (nM)																		Total amount (nM)			*p*-value		
		-12h - 0h			0h – 1h			1h – 2h			2h – 4h			4h – 6h			6h – 24h			0h – 24 h			Treat-ment	Time	Inter-action
Benzoic acid aglycones																									
2,4-dihydroxy benzoic acid	Oats	17.0	±	11.7	19.1	±	10.3	156.4	±	150.9	24.1	±	10.8	4.6	±	4.6	16.3	±	11.0	238	±	24	.	.	.
	Control	21.9	±	11.6	20.5	±	11.1	7.2	±	7.2	45.8	±	18.9	8.1	±	8.1	9.4	±	6.4	113	±	6			
2,5-dihydroxy benzoic acid	Oats	45.7	±	25.2	54.1	±	36.7	371.8	±	355.6	26.8	±	14.3	52.6	±	39.2	61.4	±	27.1	612	±	54	.	.	.
	Control	17.4	±	11.8	43.7	±	31.3	7.7	±	7.7	55.3	±	29.7	22.7	±	22.7	27.6	±	18.8	174	±	7			
3,5-dihydroxy benzoic acid	Oats	14.6	±	14.6	14.1	±	14.1	240.8	±	240.8	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	269	±	39	.	.	.
	Control	0.0	±	0.0	14.9	±	14.9	0.0	±	0.0	23.3	±	15.8	15.9	±	15.9	0.0	±	0.0	54	±	4			
4-hydroxy benzoic acid	Oats	641.9	±	104.7	626.6	±	130.8	742.5	±	236.5	526.4	±	103.4	551.7	±	131.5	592.2	±	190.7	3681	±	31	0.2187	0.5601	0.4005
	Control	507.6	±	63.8	575.5	±	88.1	526.5	±	78.7	564.8	±	83.6	555.9	±	80.3	493.7	±	64.2	3224	±	13			
syringic acid	Oats	28.4	±	19.5	25.4	±	23.5	440.9	±	436.4	11.8	±	6.7	1.9	±	0.9	29.7	±	20.3	538	±	70	0.8127	0.5049	0.6595
	Control	8.0	±	4.0	26.3	±	18.8	6.3	±	5.3	29.0	±	15.7	17.1	±	16.4	27.8	±	22.5	114	±	4			
syringaldehyde	Oats	90.5	±	58.5	65.6	±	65.6	1032.9	±	997.4	27.7	±	15.9	1.6	±	1.6	78.6	±	46.9	1297	±	164	0.9015	0.3328	0.445
	Control	42.6	±	20.3	93.1	±	59.5	14.0	±	13.1	144.8	±	76.3	100.5	±	69.5	63.5	±	38.5	458	±	19			
vanillic acid	Oats	22.8	±	16.0	16.8	±	16.8	204.9	±	194.7	0.0	±	0.0	1342.7	±	302.4	19.8	±	13.4	1607	±	217	.	0.57	0.9382
	Control	34.8	±	18.7	36.7	±	19.9	11.9	±	11.9	35.9	±	19.9	1047.1	±	232.8	18.4	±	12.4	1185	±	170			
isovanillic acid	Oats	16.3	±	11.5	11.1	±	11.1	181.8	±	178.5	5.7	±	3.9	0.0	±	0.0	12.7	±	8.6	228	±	29	.	.	.
	Control	6.8	±	4.6	14.9	±	11.0	0.0	±	0.0	21.7	±	11.6	12.0	±	9.5	11.2	±	6.1	66	±	3			
Benzoic acid sulfates																									
benzoic acid-O-sulfate	Oats	165.6	±	80.6	75.6	±	52.2	925.8	±	869.0	67.6	±	26.9	23.9	±	17.7	82.8	±	46.6	1341	±	142	0.3255	0.4708	0.6391
	Control	84.5	±	33.1	107.1	±	58.6	29.4	±	17.3	145.5	±	66.5	80.9	±	45.7	88.9	±	39.2	536	±	15			
3,4-dihydroxybenzoic acid-3-O-sulfate	Oats	40.5	±	12.0	47.0	±	9.4	364.5	±	328.0	41.0	±	11.3	1863.8	±	396.6	32.8	±	9.7	2390	±	298	0.2098	<.0001	0.7519
	Control	67.3	±	27.4	36.6	±	14.2	43.2	±	15.4	34.2	±	15.0	1563.0	±	267.8	31.6	±	10.5	1776	±	253			
3,5-dihydroxybenzoic acid-3-O-sulfate	Oats	9.2	±	6.2	14.2	±	14.1	313.3	±	310.3	2.2	±	1.7	0.3	±	0.3	7.2	±	5.9	346	±	51	0.1858	0.0002	0.0008
	Control	5.9	±	4.0	6.1	±	5.3	0.5	±	0.4	4.8	±	2.5	2.5	±	1.5	7.4	±	5.4	27	±	1			

p-salicyclic acid-4-sulfate	Oats	76.6	±	34.7	39.4	±	22.8	448.1	±	420.7	30.5	±	9.7	12.3	±	6.8	38.1	±	20.0	645	±	69	0.2515	0.4868	0.2746
	Control	39.0	±	13.7	56.2	±	25.6	17.4	±	7.2	58.0	±	25.4	47.4	±	23.9	44.9	±	18.4	263	±	6			
syringaldehyde-O-sulfate	Oats	127.3	±	56.1	163.1	±	76.5	270.1	±	142.3	98.7	±	49.3	112.4	±	54.0	79.7	±	22.2	851	±	28	0.7496	0.5057	0.3257
	Control	210.9	±	94.3	262.8	±	112.2	91.7	±	27.4	105.3	±	56.9	173.7	±	62.1	147.5	±	43.9	992	±	26			
Benzoic acid glucuronides																									
salicylic acid-B-D-O-glucuronide	Oats	36.7	±	18.4	10.9	±	10.9	294.7	±	276.8	17.2	±	9.9	4.6	±	4.6	15.0	±	10.4	379	±	47	0.9256	0.9821	0.977
	Control	18.5	±	9.8	33.0	±	18.2	0.0	±	0.0	20.3	±	10.8	25.8	±	17.8	22.4	±	10.4	120	±	5			
Phenylacetic acid aglycones																									
4-hydroxyphenylacetic acid	Oats	798.5	±	219.0	432.2	±	217.7	1137.4	±	591.1	345.9	±	174.7	353.6	±	242.3	321.9	±	161.2	3390	±	136	0.0201	0.0821	0.0966
	Control	280.3	±	191.7	872.3	±	316.1	729.2	±	259.0	1066.3	±	300.1	745.7	±	394.2	314.5	±	220.8	4008	±	127			
Phenylacetic acid sulfates																									
4-hydroxyphenylacetic acid-O-sulfate	Oats	58.4	±	58.4	0.0	±	0.0	59.3	±	41.1	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	118	±	12	.	.	.
	Control	25.0	±	25.0	42.8	±	42.8	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	68	±	8			
homovanillic acid sulfate	Oats	45.6	±	24.2	27.6	±	19.6	371.5	±	360.4	15.7	±	5.8	5.3	±	2.3	32.7	±	17.1	498	±	58	0.3621	0.7655	0.48
	Control	25.5	±	8.6	37.8	±	21.9	13.3	±	5.4	47.3	±	21.4	26.0	±	11.7	33.6	±	15.4	184	±	5			
Hippuric acid aglycones																									
hippuric acid	Oats	1914.8	±	732.0	1284.9	±	631.2	1643.5	±	687.7	1025.4	±	333.2	1290.8	±	441.7	1029.9	±	209.8	8189	±	144	0.8684	0.6569	0.4595
	Control	1177.6	±	253.0	1392.3	±	305.8	870.2	±	105.5	985.7	±	207.5	1129.6	±	191.0	1085.6	±	248.6	6641	±	73			
2-hydroxyhippuric acid	Oats	64.7	±	27.4	37.2	±	19.0	283.5	±	260.9	20.5	±	8.1	27.4	±	6.0	65.8	±	28.7	499	±	41	0.1847	0.3636	0.3303
	Control	45.0	±	13.9	52.8	±	21.9	20.8	±	6.2	52.2	±	21.6	37.7	±	17.2	30.3	±	13.2	239	±	5			
3-hydroxyhippuric acid	Oats	76.1	±	36.0	51.1	±	28.4	200.6	±	162.6	41.2	±	19.3	13.7	±	6.6	37.6	±	20.6	420	±	27	0.2604	0.2229	0.4806
	Control	45.7	±	18.7	57.3	±	24.5	27.8	±	17.4	48.0	±	23.0	43.3	±	18.6	40.3	±	21.4	262	±	4			
4-hydroxyhippuric acid	Oats	149.1	±	44.9	118.1	±	34.7	364.9	±	266.6	83.6	±	22.5	90.3	±	18.1	105.6	±	27.3	912	±	44	0.2752	0.6256	0.413
	Control	119.1	±	28.7	127.3	±	28.7	62.8	±	20.8	111.4	±	35.7	79.8	±	20.6	138.7	±	29.2	639	±	12			
Hippuric acid sulfates																									
hippuric acid-O-sulfate	Oats	25.6	±	11.6	18.7	±	9.8	18.8	±	10.8	16.7	±	8.3	18.4	±	9.3	18.4	±	8.6	117	±	1	0.279	0.3542	0.576
	Control	14.3	±	5.3	15.8	±	6.1	11.6	±	5.3	10.8	±	4.7	13.3	±	5.3	9.7	±	4.5	76	±	1			
Cinnamic acid aglycones																									
caffeic acid	Oats	76.8	±	18.7	51.7	±	23.6	85.2	±	30.6	37.2	±	17.0	64.2	±	24.2	39.5	±	11.2	355	±	8	0.4437	0.4099	0.2222

	Control	64.7	±	14.5	67.5	±	18.4	46.1	±	17.5	55.2	±	18.8	65.4	±	18.8	74.4	±	15.6	373	±	4			
ferulic acid	Oats	10.6	±	7.8	33.9	±	17.5	153.5	±	145.2	8.5	±	2.7	3.5	±	1.7	11.8	±	5.6	222	±	24	0.7408	0.8658	0.9615
	Control	6.4	±	2.4	9.0	±	6.4	10.3	±	9.3	11.6	±	6.0	13.9	±	7.9	6.9	±	3.3	58	±	1	.	.	.
isoferulic acid	Oats	89.1	±	56.5	135.3	±	71.3	942.3	±	892.3	36.4	±	19.8	5.6	±	3.4	57.5	±	29.8	1266	±	147	0.4831	0.4781	0.6443
	Control	37.2	±	16.7	52.4	±	32.9	50.2	±	38.2	104.5	±	57.8	74.7	±	38.3	51.0	±	24.7	370	±	10			
hydroferulic acid	Oats	16.2	±	7.7	8.2	±	4.7	118.1	±	112.1	6.0	±	2.6	23.5	±	6.4	22.5	±	7.7	195	±	17	0.2045	0.6931	0.4281
	Control	16.7	±	4.2	18.3	±	6.5	7.1	±	3.2	16.0	±	8.0	9.1	±	5.2	18.8	±	5.2	86	±	2			
p-coumaric acid	Oats	12.5	±	7.6	8.2	±	8.2	152.3	±	150.1	5.5	±	3.0	0.0	±	0.0	9.1	±	6.2	188	±	24	.	0.0364	0.0225
	Control	6.9	±	3.0	10.5	±	7.7	0.0	±	0.0	20.6	±	8.7	9.8	±	7.8	10.9	±	5.3	59	±	3			
sinapic acid	Oats	0.0	±	0.0	0.0	±	0.0	2.1	±	2.1	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	2	±	0	.	.	.
	Control	0.0	±	0.0	0.1	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.2	0.1	±	0.1	1	±	0			
Cinnamic acid sulfates																									
caffeic acid-O-sulfate	Oats	0.9	±	0.9	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	1.5	±	1.5	0.0	±	0.0	2	±	0	.	.	.
	Control	1.0	±	1.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	1	±	0			
dihydro caffeic acid 3-O-sulfate	Oats	32.5	±	20.9	22.9	±	19.4	345.2	±	337.4	12.1	±	5.9	2.2	±	1.7	25.6	±	14.9	440	±	55	0.6429	0.2535	0.6156
	Control	17.9	±	8.2	30.8	±	20.0	8.0	±	4.3	41.3	±	21.1	26.4	±	16.3	19.2	±	9.6	143	±	5			
ferulic acid-4-0-sulfate	Oats	30.6	±	22.1	18.9	±	18.9	302.8	±	294.6	0.0	±	0.0	0.0	±	0.0	19.0	±	12.9	371	±	48	.	.	.
	Control	8.1	±	8.1	26.4	±	18.2	0.0	±	0.0	35.4	±	19.3	14.7	±	14.7	11.6	±	11.6	96	±	5			
dihydroferulic acid 4-O-sulfate	Oats	36.5	±	19.9	20.0	±	17.2	317.8	±	309.7	13.0	±	6.0	6.0	±	2.6	29.7	±	15.9	423	±	50	0.3658	0.5162	0.4652
	Control	21.2	±	8.2	34.0	±	18.9	12.5	±	4.1	37.3	±	18.9	25.6	±	16.0	29.4	±	13.5	160	±	4			
isoferulic acid-3-O-sulfate	Oats	18.3	±	12.5	21.8	±	21.8	398.9	±	398.9	0.0	±	0.0	0.0	±	0.0	14.4	±	14.4	453	±	65	.	.	.
	Control	7.6	±	7.6	27.8	±	21.8	0.0	±	0.0	40.0	±	22.9	22.2	±	22.2	14.5	±	14.5	112	±	6			
p-coumaric acid 4-O-sulfate	Oats	21.8	±	14.3	14.2	±	14.2	281.9	±	277.7	6.7	±	3.6	0.4	±	0.4	22.7	±	13.4	348	±	45	0.8279	0.1715	0.4567
	Control	10.5	±	5.0	22.3	±	15.4	4.7	±	3.3	26.9	±	14.1	19.2	±	13.7	16.1	±	10.6	100	±	3			
sinapic acid-O-sulfate	Oats	2.3	±	2.3	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	2	±	0	.	.	.
	Control	2.0	±	2.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	2	±	0			
Cinnamic acid glucuronides																									
caffeic acid 3-B-D-glucuronide	Oats	15.5	±	10.0	10.7	±	10.6	219.0	±	212.0	8.0	±	4.3	1.9	±	1.8	12.7	±	7.3	268	±	35	0.6472	0.4077	0.445
	Control	5.2	±	2.5	13.6	±	9.1	2.0	±	1.4	16.9	±	9.6	14.2	±	9.7	12.5	±	7.2	64	±	2			

caffeic acid 4-B-D-glucuronide	Oats	39.5	±	23.5	25.2	±	23.9	361.5	±	351.7	16.7	±	7.5	3.1	±	1.4	33.3	±	18.0	479	±	57	0.2518	0.2663	0.7215
	Control	20.1	±	9.2	34.7	±	21.4	9.4	±	4.5	47.4	±	24.7	30.1	±	18.0	30.8	±	17.7	172	±	5			
dihydroferulic acid glucuronide	Oats	85.6	±	40.5	31.7	±	31.7	451.4	±	437.9	18.4	±	12.5	21.4	±	15.4	47.0	±	26.4	655	±	69	0.8554	0.2649	0.5289
	Control	35.9	±	19.9	59.8	±	34.9	16.8	±	11.5	52.8	±	28.6	33.9	±	27.3	57.9	±	26.9	257	±	7			
ferulic acid-4-B-D-G-glucuronide	Oats	12.5	±	5.9	49.7	±	17.9	269.0	±	234.6	34.1	±	7.1	10.6	±	2.8	17.0	±	8.4	393	±	41	0.7414	0.7044	0.8318
	Control	12.7	±	5.4	18.1	±	10.9	6.0	±	1.8	33.9	±	18.1	23.4	±	13.8	16.2	±	8.9	110	±	4			
dihydro isoferulic acid 3-O-B-D	Oats	53.3	±	32.5	29.7	±	27.1	459.8	±	449.8	16.6	±	9.1	2.8	±	2.8	39.4	±	22.3	602	±	72	0.6649	0.2796	0.6295
-glucuronide	Control	23.8	±	10.7	41.0	±	29.6	4.4	±	4.4	52.3	±	28.4	28.5	±	22.4	36.5	±	19.5	187	±	7			
p-coumaric acid glucuronide	Oats	591.6	±	85.7	618.6	±	99.1	530.2	±	72.5	547.3	±	78.1	582.4	±	108.4	599.0	±	96.0	3469	±	14	0.0798	0.5243	0.7962
	Control	523.4	±	70.8	604.5	±	72.1	599.4	±	76.2	581.5	±	73.1	640.7	±	94.5	578.1	±	71.5	3528	±	16			
Benzaldehydes																									
vanillin	Oats	25.3	±	17.2	12.2	±	12.2	262.7	±	262.7	0.0	±	0.0	14.7	±	14.7	11.5	±	11.5	326	±	42	.	.	.
	Control	0.0	±	0.0	27.8	±	19.2	0.0	±	0.0	12.3	±	12.3	17.6	±	17.6	0.0	±	0.0	58	±	5			
Avenanthramides																									
Avenanthramide A	Oats	27.5	±	16.8	22.0	±	19.1	400.1	±	391.4	14.3	±	6.1	2.2	±	1.3	27.7	±	16.1	494	±	64	0.8099	0.4344	0.4499
	Control	15.1	±	6.8	27.7	±	19.7	5.6	±	4.4	38.1	±	20.6	23.2	±	15.6	22.9	±	13.7	133	±	5			
Avenanthramide B	Oats	18.1	±	11.5	10.7	±	10.7	247.8	±	244.3	7.0	±	3.9	0.2	±	0.2	16.3	±	9.8	300	±	40	0.5522	0.35	0.7614
	Control	6.1	±	2.8	20.7	±	14.0	3.4	±	2.7	26.5	±	14.3	15.4	±	11.6	14.4	±	8.9	87	±	4			
Avenanthramide C	Oats	2.1	±	2.1	1.7	±	1.7	47.2	±	47.2	1.2	±	0.9	0.0	±	0.0	3.6	±	2.5	56	±	8	.	.	.
	Control	0.5	±	0.5	4.2	±	3.5	0.7	±	0.7	5.4	±	2.9	3.0	±	2.5	1.4	±	1.0	15	±	1			
Total concentration																									
	Oats	5685.0	±	47.6	4300.0	±	34.1	16416.6	±	48.7	3217.6	±	28.0	6518.2	±	58.6	3737.5	±	28.6	39875.0	±	211.7	0.5228	0.4112	0.8523
	Control	3640.7	±	30.1	5077.0	±	38.7	3278.1	±	29.4	4866.4	±	34.5	6859.2	±	50.9	3670.6	±	28.7	27392.0	±	192.9			

Table 3.6 Concentrations of individual compounds in urine following the intake of oats and control. **Urinary excretion rate per hour of phenolic acids and avenanthramides (µg). Values are expressed as means ± SEMs.Significance was tested with using a linear mixed model with Bonferroni corrections (n=14).**

		Phenolic acid concentration (µg)												Total amount (µg)			p-value		
		-12h - 0h			0h – 3h			3h – 7h			7h – 24h			0h – 24 h			Treat-ment	Time	Inter-action
Benzoic acid aglycones																			
2,4-dihydroxy benzoic acid	Oats	7.1	±	1.8	45.8	±	13.3	19.0	±	5.9	17.7	±	3.3	89.6	±	8.2	0.60	0.01	0.02
	Control	7.5	±	1.9	34.4	±	8.8	24.4	±	5.4	11.4	±	2.7	77.7	±	6.2			
2,5-dihydroxy benzoic acid	Oats	10.6	±	2.0	95.8	±	14.1	79.5	±	12.8	125.4	±	19.7	311.2	±	24.3	0.01	0.07	0.01
	Control	10.6	±	1.3	67.0	±	12.6	46.7	±	7.8	33.2	±	5.1	157.5	±	11.9			
3,5-dihydroxy benzoic acid	Oats	7.6	±	1.6	41.8	±	7.4	19.7	±	3.9	21.3	±	4.4	90.3	±	7.1	0.39	0.01	0.06
	Control	6.9	±	1.0	37.0	±	4.7	27.6	±	5.0	18.9	±	3.6	90.4	±	6.4			
4-hydroxy benzoic acid	Oats	18.3	±	3.7	103.2	±	15.0	53.8	±	8.4	47.0	±	9.6	222.3	±	17.6	0.38	0.0002	0.23
	Control	13.1	±	1.7	105.7	±	18.3	76.5	±	10.7	39.3	±	7.2	234.6	±	20.4			
syringic acid	Oats	1.4	±	1.0	26.7	±	6.6	12.2	±	3.1	12.0	±	2.3	52.5	±	5.2	0.0009	0.1111	0.1035
	Control	1.0	±	0.3	3.1	±	1.8	2.2	±	1.1	5.2	±	1.2	11.5	±	0.9			
syringaldehyde	Oats	0	±	0	0	±	0	0	±	0	0	±	0	0.1	±	0.0	.	.	.
	Control	0	±	0	0	±	0	0	±	0	0	±	0	0.1	±	0.0			
vanillic acid	Oats	5.6	±	2.5	83.0	±	28.0	111.3	±	33.6	50.9	±	12.8	250.8	±	22.7	0.05	0.01	0.23
	Control	4.6	±	1.2	66.3	±	31.9	213.5	±	57.4	52.2	±	14.7	336.7	±	45.1			
isovanillic acid	Oats	0.1	±	0.1	1.4	±	0.8	2.6	±	1.2	1.8	±	0.4	6.0	±	0.5	0.1563	0.0096	0.029
	Control	0.1	±	0	1.4	±	1.1	3.2	±	1.3	1.6	±	0.4	6.2	±	0.6			
Benzoic acid sulfates																			
benzoic acid-O-sulfate	Oats	181.3	±	35.9	1063.0	±	194.8	659.2	±	134.4	681.7	±	145.7	2585.2	±	180.6	0.51	0.0015	0.28
	Control	190.2	±	32.6	917.6	±	181.8	736.3	±	155.3	570.4	±	114.1	2414.5	±	155.0			
3,4-dihydroxybenzoic acid-3-O-sulfate	Oats	5.1	±	1.0	178.8	±	85.0	178.7	±	109.3	146.5	±	91.2	509.2	±	41.4	0.36	0.96	0.81
	Control	19.4	±	7.8	185.6	±	106.1	146.8	±	111.3	204.5	±	120.6	556.2	±	41.7			
3,5-dihydroxybenzoic acid-3-O-sulfate	Oats	13.5	±	2.6	68.1	±	11.3	32.7	±	6.8	38.9	±	7.2	153.3	±	11.3	0.79	0.0003	0.1216

	Control	14.9	±	2.5	64.3	±	10.3	45.2	±	7.3	36.2	±	7.0	160.6	±	10.2			
p-salicyclic acid-4-sulfate	Oats	95.6	±	19.2	546.5	±	101.2	340.8	±	69.2	379.0	±	79.2	1361.9	±	93.0	0.29	0.0011	0.34
	Control	94.7	±	15.0	470.8	±	95.2	375.3	±	77.3	295.7	±	60.9	1236.5	±	79.9			
syringic acid-O-sulfate	Oats	0	±	0	0.1	±	0.1	0.1	±	0.1	0.3	±	0.1	0.5	±	0.1	.	.	.
	Control	0	±	0	0.1	±	0.1	0	±	0	0.7	±	0.2	0.8	±	0.2			
syringaldehyde-O-sulfate	Oats	119.6	±	21.2	902.2	±	113.0	459.0	±	85.3	414.8	±	55.5	1895.5	±	161.4	0.6547	0.0001	0.34
	Control	164.8	±	35.7	836.4	±	114.0	593.6	±	90.9	362.2	±	54.0	1957.0	±	145.1			
Benzoic acid glucuronides																			
salicylic acid-B-D-O-glucuronide	Oats	10.4	±	5.7	18.4	±	8.2	7.6	±	2.6	10.2	±	2.9	46.5	±	2.3	0.39	0.16	0.23
	Control	1.7	±	0.5	12.4	±	2.8	6.7	±	1.4	3.7	±	1.3	24.6	±	2.3			
Phenylacetic acid aglycones																			
4-hydroxyphenylacetic acid	Oats	48.2	±	8.4	394.7	±	140.2	334.8	±	170.8	133.5	±	32.9	911.2	±	81.9	0.65	0.02	0.88
	Control	132.0	±	60.9	419.2	±	208.3	317.5	±	108.3	183.7	±	53.8	1052.4	±	65.1			
homovanillic acid	Oats	7.4	±	3.6	55.1	±	37.8	8.0	±	8.0	135.7	±	61.0	206.1	±	30.2	.	.	.
	Control	18.7	±	8.1	72.4	±	39.4	47.8	±	26.2	118.1	±	36.2	257.0	±	21.0			
Phenylacetic acid sulfates																			
4-hydroxyphenylacetic acid-O-sulfate	Oats	14.3	±	5.6	85.6	±	19.4	39.0	±	11.3	33.7	±	8.8	172.6	±	15.1	0.47	0.0002	0.46
	Control	15.1	±	4.6	49.8	±	17.2	38.0	±	11.2	29.5	±	11.5	132.4	±	7.3			
homovanillic acid sulfate	Oats	33.1	±	5.6	155.8	±	19.8	78.2	±	13.9	126.9	±	22.5	394.0	±	27.1	0.4132	0.0008	0.3509
	Control	34.3	±	4.2	159.8	±	27.7	111.2	±	14.9	124.9	±	24.4	430.3	±	26.5			
Hippuric acid aglycones																			
hippuric acid	Oats	458.8	±	89.5	2764.6	±	588.8	1442.3	±	248.5	1348.1	±	213.8	6013.8	±	475.2	0.71	0.0005	0.01
	Control	393.7	±	51.7	2474.4	±	305.3	1796.4	±	242.5	993.4	±	152.6	5657.9	±	455.4			
2-hydroxyhippuric acid	Oats	64.3	±	26.0	199.1	±	60.0	91.9	±	19.3	151.1	±	27.5	506.4	±	30.2	0.236	0.0113	0.0195
	Control	24.5	±	3.8	143.1	±	28.0	87.1	±	12.6	62.1	±	10.0	316.9	±	24.9			
3-hydroxyhippuric acid	Oats	28.5	±	6.9	170.6	±	45.0	83.3	±	16.5	101.9	±	15.3	384.3	±	29.3	0.7313	0.0069	0.0558
	Control	27.1	±	98.9	199.5	±	45.1	121.5	±	26.3	57.7	±	11.1	405.8	±	38.1			
4-hydroxyhippuric acid	Oats	98.4	±	16.0	614.1	±	69.5	334.4	±	51.8	281.1	±	39.0	1328.1	±	106.8	0.90	0.0001	0.4782
	Control	4.5	±	12.8	555.0	±	74.6	386.0	±	46.3	309.9	±	54.8	1255.5	±	115.1			

Hippuric acid sulfates																		
hippuric acid-O-sulfate	Oats	10.2	±	4.5	73.9	±	35.9	36.6	±	17.0	19.7	±	5.7	140.4	±	14.0	0.89	0.12	0.39
	Control	8.8	±	3.0	46.0	±	11.4	48.8	±	15.8	22.4	±	7.0	126.0	±	9.6			
Cinnamic acid aglycones																		
caffeic acid	Oats	17.7	±	3.5	106.6	±	22.6	55.8	±	9.7	50.9	±	7.9	231.0	±	18.3	0.51	0.0008	0.11
	Control	15.4	±	2.2	95.6	±	11.4	68.5	±	9.0	37.5	±	5.6	217.0	±	17.6			
ferulic acid	Oats	1.9	±	0.7	24.2	±	5.5	11.9	±	2.6	16.0	±	4.8	54.0	±	4.6	0.01	0.0233	0.64
	Control	1.4	±	0.3	9.9	±	3.4	5.7	±	2.0	9.0	±	3.7	26.0	±	1.9			
isoferulic acid	Oats	4.1	±	1.0	57.8	±	14.6	22.3	±	5.2	26.7	±	8.9	110.9	±	11.2	0.10	0.04	0.28
	Control	4.4	±	1.3	33.1	±	9.6	19.1	±	5.5	11.1	±	2.6	67.7	±	6.2			
hydroferulic acid	Oats	2.5	±	0.8	16.0	±	3.9	7.4	±	2.1	7.0	±	2.3	32.9	±	2.8	0.65	0.01	0.29
	Control	3.1	±	0.8	21.9	±	5.5	10.8	±	2.5	3.2	±	1.3	39.0	±	4.4			
p-coumaric acid	Oats	0.3	±	0.1	2.2	±	0.6	0.9	±	0.2	0.8	±	0.2	4.1	±	0.4	0.83	0.08	0.30
	Control	0.2	±	0	1.2	±	0.2	1.0	±	0.2	0.8	±	0.4	3.3	±	0.2			
sinapic acid	Oats	0.7	±	0.2	13.6	±	2.4	5.3	±	1.2	8.0	±	2.2	27.5	±	2.7	.	.	.
	Control	0.8	±	0.2	3.7	±	1.4	2.2	±	0.7	5.8	±	3.0	12.5	±	1.1			
Cinnamic acid sulfates																		
caffeic acid-O-sulfate	Oats	5.1	±	1.3	97.5	±	24.0	112.0	±	42.9	40.5	±	7.7	255.0	±	24.9	0.02	0.04	0.17
	Control	7.9	±	3.2	35.5	±	14.2	19.5	±	4.9	20.9	±	5.1	83.8	±	5.7			
dihydro caffeic acid 3-O-sulfate	Oats	17.9	±	7.0	100.1	±	28.9	43.1	±	15.3	85.2	±	27.2	246.3	±	18.9	0.54	0.12	0.24
	Control	16.0	±	4.9	104.7	±	39.8	70.7	±	21.3	64.3	±	30.4	255.7	±	18.3			
ferulic acid-4-0-sulfate	Oats	24.8	±	6.1	873.5	±	156.5	539.7	±	128.3	251.9	±	47.4	1689.8	±	183.6	0.236	0.01	0.03
	Control	36.3	±	8.0	188.7	±	40.1	119.1	±	18.8	210.3	±	51.0	554.4	±	39.3			
dihydroferulic acid 4-O-sulfate	Oats	11.0	±	3.5	64.0	±	15.4	29.1	±	7.9	61.8	±	14.2	166.0	±	12.9	0.7824	0.0006	0.17
	Control	15.4	±	3.2	83.4	±	23.4	40.1	±	10.0	35.0	±	7.6	173.9	±	14.3			
isoferulic acid-3-O-sulfate	Oats	1.1	±	0.6	41.9	±	10.7	26.6	±	6.9	6.8	±	1.7	76.3	±	9.4	0.0121	0.03	0.18
	Control	3.0	±	1.1	10.7	±	4.7	4.7	±	2.3	5.5	±	1.7	23.9	±	1.6			
p-coumaric acid 4-O-sulfate	Oats	1.5	±	0.5	5.7	±	1.1	3.0	±	0.6	2.9	±	1.0	13.0	±	0.9	0.63	0.0039	0.35
	Control	0.9	±	0.3	3.7	±	1.0	3.5	±	1.0	5.7	±	2.5	13.7	±	1.0			

sinapic acid-O-sulfate	Oats	21.5	±	6.2	450.6	±	59.8	241.5	±	48.6	234.3	±	69.6	948.0	±	87.6	0.01	0..22	0.02
	Control	32.0	±	9.5	105.6	±	24.5	64.5	±	11.6	151.3	±	33.9	353.4	±	25.8			
Cinnamic acid glucuronides																			
caffeic acid 3-B-D-glucuronide	Oats	0.9	±	0.3	7.2	±	1.3	5.7	±	1.2	4.6	±	2.0	18.4	±	1.4	0.02	0.07	0.85
	Control	1.0	±	0.3	3.3	±	1.3	2.2	±	0.9	3.1	±	1.3	9.6	±	0.5			
caffeic acid 4-B-D-glucuronide	Oats	0.6	±	0.3	8.2	±	1.2	5.6	±	1.0	2.0	±	0.5	16.4	±	1.7	0.00	0.0001	0.36
	Control	0.5	±	0.2	5.1	±	1.5	3.1	±	1.0	1.2	±	0.4	9.9	±	1.0			
dihydroferulic acid glucuronide	Oats	9.8	±	2.4	54.8	±	10.6	30.0	±	8.9	47.8	±	10.6	142.5	±	10.1	0.739	0.01	0.10
	Control	13.4	±	2.6	80.8	±	16.6	41.4	±	8.6	30.3	±	7.5	165.9	±	14.3			
ferulic acid-4-B-D-G-glucuronide	Oats	8.4	±	2.3	161.6	±	24.8	124.3	±	28.6	61.0	±	9.2	355.4	±	33.9	0.06	0.01	0.12
	Control	12.4	±	3.0	69.8	±	14.8	44.2	±	6.2	46.7	±	9.7	173.1	±	11.8			
dihydro isoferulic acid 3-O-B-D	Oats	10.5	±	1.9	56.7	±	11.6	23.5	±	5.1	48.8	±	11.5	139.6	±	10.8	0.85	0.0006	0.08
-glucuronide	Control	11.4	±	1.8	60.4	±	14.8	29.1	±	5.3	27.0	±	4.8	127.9	±	10.3			
p-coumaric acid glucuronide	Oats	4.3	±	1.1	40.0	±	6.6	20.4	±	3.6	4.9	±	1.1	69.6	±	8.4	0.27	0.0001	0.29
	Control	3.8	±	0.9	33.8	±	6.9	24.2	±	3.8	3.7	±	0.7	65.5	±	7.6			
Benzaldehydes																			
vanillin	Oats	1.0	±	0.2	9.6	±	1.1	5.8	±	1.6	5.4	±	0.8	21.8	±	1.7	0.54	0.0001	0.52
	Control	1.4	±	0.2	10.5	±	1.9	8.3	±	1.4	4.5	±	0.7	24.7	±	2.0			
Avenanthramides																			
Avenanthramide A	Oats	0.2	±	0	3.7	±	0.8	2.8	±	0.6	1.1	±	0.2	7.9	±	0.8	0.0016	0.0005	0.01
	Control	0.3	±	0	1.5	±	0.3	0.9	±	0.2	0.8	±	0.2	3.5	±	0.2			
Avenanthramide B	Oats	0	±	0	0.2	±	0.1	0.2	±	0	0	±	0	0.4	±	0.1	.	.	.
	Control	0	±	0	0	±	0	0	±	0	0	±	0	0.0	±	0.0			
Avenanthramide C	Oats	0	±	0	0.1	±	0.1	0.1	±	0	0.1	±	0	0.3	±	0.0	.	.	.
	Control	0	±	0	0	±	0	0	±	0	0	±	0	0.0	±	0.0			
Total concentration (µg)																			
	Oats	1380.3	±	10.9	9786.9	±	67.9	5629.5	±	37.0	5207.6	±	33.8	22004	±	148.5	0.22	0.08	0.71
	Control	1361.4	±	10.1	7848.6	±	59.3	5815.6	±	43.6	4194.1	±	26.4	19220	±	138.3			

3.4.4 Serum metabolite levels predict improvements in endothelium independent microvascular function

Analysis of phenolic metabolites indicated a large variability in terms of absorption between study volunteers (**Figure 3.7 A and B**) with some volunteers classified as absorbers and others low/non-absorbers, 2h post consumption of oats. Endothelium independent microvascular function was found close to be significantly higher in those individuals who were classified as absorbers compared to those classified as non-absorbers (**Figure 3.7 C**; p value<0.06). Correlation analysis indicated that the blood levels of salicyclic acid sulphate (ρ=0.38, p=0.04), syringic acid (ρ=0.38, p=0.04), ferulic acid sulfate (ρ=0.41, p=0.02), 2,5-dihydroxybenzoic acid (ρ=0.41, p=0.02), p-coumaric acid (ρ=0.41, p=0.02), isovanillic acid (ρ=0.41, p=0.02) , salicyclic acid glucuronide (ρ=0.45, p=0.01) and ferulic acid glucuronide (ρ=0.50, p=0.01) in volunteers at 2h significantly correlated with endothelium independent microvascular function (**Table 3.7**).

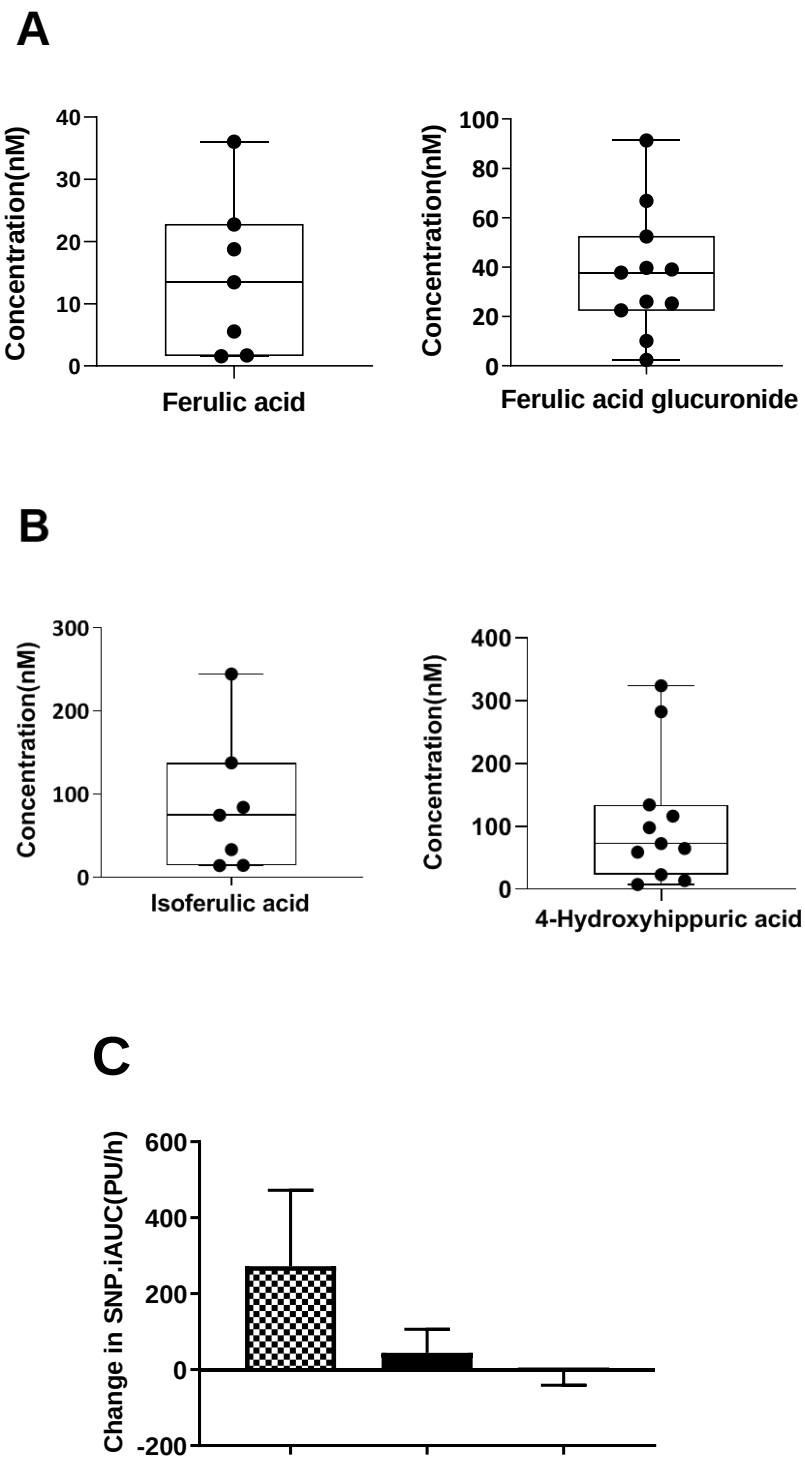

Figure 3.7 Inter-individual variability of absorption between individuals and change in microvascular function.

Examples of main phenolic acids showing inter-individual variability in absorption (**A** and **B**) and change in microvascular function in relation to high (squared bar ,n=3) , all (filled bar, n=11) and low/non absorbers (lined bar, n=3) (**C**). One volunteer was very high absorber for all phenolics and excluded when plotting the data to see the distribution of others volunteers in **A** and **B**. Data expressed as area under the curve (AUC) of serum concentration (nM) for panel **A** and **B** and as means ± SEMs for SNP.iAUC (PU/h) for panel **C**. For panel C, significance was tested with an unpaired t-test at a significance level ($p < 0.05$).

Table 3.7 Correlation analysis between metabolites and endothelium independent microvascular function. Significant correlations ($p < 0.05$) between serum derived phenolic metabolites and changes in endothelium independent microvascular function (SNP.iAUC) at 2 hours after consumption of oats (n=13). Correlations were tested with Spearman's ρ.

Metabolites correlating with SNP.iAUC at 2h	Spearman's ρ	p-value
Salicyclic acid sulfate	0.38	0.04
Syringic acid	0.38	0.04
Ferulic acid sulfate	0.41	0.02
2,5 dihydroxybenzoic acid	0.41	0.02
P-coumaric acid	0.41	0.02
Isovanillic acid	0.41	0.02
Salicyclic acid glucuronide	0.45	0.01
Ferulic acid glucuronide	0.5	0.01

3.5 Discussion

This study aimed to examine whether the consumption of a single intake of oats containing phenolic acids and avenanthramides would acutely effect the cardiovascular risk biomarkers including endothelial function, microvascular blood flow , BP and NADPH oxidase activity in men with pre- and stage 1 hypertension. Additionally, pharmacokinetics of serum and urine phenolic metabolites and their associations with cardiovascular risk biomarkers were tested. Our data indicated an intake of phenolic acids and avenanthramides (50mg) led to an increase in both endothelium-dependent and endothelium-independent microvascular dilation at 2h, although not on FMD or BP, relative to control. Although similar clinical datasets that attempt to isolate the biological impact of phenolic acids and avenanthramides in oats are rare, data exist for the impact of other phenolic-containing foods and beverages on similar outcomes (29, 30, 202, 283, 312, 327). Our study is broadly in agreement with other clinical trials which investigate the effects of consumption of wholegrain oats on vasodilation (278, 280, 328, 329), although our data show weaker associations in relation to FMD. For example, oat bread intake providing 30 g β-glucan per day 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 and also increased the NO levels(278) and whole grain oat or wheat cereals have been observed to have no effect on brachial artery vasodilatation chronically when consumed with a high fat diet(328). Conversely, chronic consumption of wild green oat extract(1500mg), is reported to significantly improve vasodilator function in healthy older adults(>60yrs)(278). It must be noted that these studies did not isolate the effect of the phenolic acids and avenanthramides from fibre and it is difficult to evaluate whether there is a synergistic effect coming together from fibre and phenolics or an improvement caused by phenolics or fibre only.

The impact of oat polyphenol intake on FMD and BP, appear to be more subtle than that observed in other studies, particularly those which administered higher doses of phenolic acids compared to our oat intervention (310 mg for coffee compared to 50 mg for oat)(30). Therefore, the relatively low oat phenolic acid intake in our study may not be sufficient to induce statistical reliable changes in FMD increase and BP as those observed for other phenolic acid- and flavonoid-containing interventions (29, 192, 283, 330-332). For example, small phenolic acids in blueberries and coffee have been shown to improve FMD that also correlated with the appearance of phenolic acid metabolites in blood (29, 30).Our microvascular vasodilation results, while showing significant responses to the intervention, also agree broadly with a randomised, placebo-controlled trial indicating that the moderate intake of champagne(375ml) providing 23.7mg of phenolic acids acutely improves endothelium-independent vasodilation at 4 and 8 h post consumption (312), particularly with respect to endothelium-independent blood flow 2 h following intake of oats. Interestingly, the doses administered in both studies (23.7 mg for champagne and 50mg for oats) were similar and sufficient to induce an increase in microvascular response.

We demonstrated the presence of 45 phenolic acids and avenanthramides in the blood. In line with the LDI results, there was a non-significant increase in serum phenolic acids, avenanthramides and their metabolites at 2h after oat consumption. This is in agreement with other studies reporting the appearance of phenolic acid metabolites and avenanthramides at 1-2h post consumption of polyphenols or wholegrain cereals, which could be explained by the rapid absorption of free and conjugated oat phenolics from the small intestine to the circulation in our study (27, 29, 30, 150, 283, 333-335). Similar to our results, structurally bigger oat avenanthramides have been repeatedly shown to be bioavailable in humans when ingested at low (21.6mg, comparatively higher than our dose (5 mg)) and high doses (75-150 mg) and exert certain cardioprotective effects in cell models and in humans (27, 119, 165, 336, 337).Oats contain a high proportion of bound phenolic acids and the bio-accessibility of these compounds are limited until the action of gut microbiota cleave the linkages between phenolic acids and fibre to yield colonic metabolites. Comparable findings of plasma pharmacokinetics of phenolic acids were also reported after the consumption of high bran wheat cereals and aleurone rich wheat bread (150, 333). The similar profile of phenolics were also detected in urine mainly peaking at 3h and slightly increasing at 24h after the consumption of oats with a total recovery % of 5.7 which is in line with other studies(150, 333) . A previous work in our group suggested an increase in mean excretion between 0-2 and 4-8h, while the results of this current study also supports the early absorption of total phenolics from the small intestine at 3h, there was no significant increase at the later timepoint (152)(See Appendix). The increase of several individual phenolic acids such as ferulic acid and its glucoronidated and sulphated metabolites at 7h in our study could be explained by role of gut microbiota on specific phenolics.

A large inter-individual variability in the absorption of oat phenolics could also explain the non-significant results at 2h in our study. Based on this variability, it was observed that volunteers defined as ‘absorbers’ showed improvements in endothelium-independent microvascular response in comparison with those defined as ‘low or non-absorbers’ . There are number of factors such as genetic polymorphisms of metabolizing enzymes and transporters and the composition of the gut microbiota which can affect the metabolism and absorption and these factors may explain the broad variability between our study volunteers (338, 339). In support of this, our data also indicated positive correlations between circulating levels of salicyclic acid sulfate, salicyclic acid glucuronide, ferulic acid sulfate, ferulic acid glucuronide, 2,5-dihydroxybenzoic acid , p-coumaric acid, isovanillic acid and syringic acid and endothelium-independent microvascular function 2h following oat consumption. Therefore, these phenolic acids may be important mediators of improvements observed in microvascular function.

A potential mechanism behind the blood vessel vasodilation mediated by circulating polyphenol metabolites is suggested to be the reduced superoxide production and increased NO bioavailability, via inhibition of NADPH oxidase (28, 29, 340, 341). The same acute intervention study discussed above

that reported improvements in FMD also reported the significant reduction in NADPH oxidase activity at 1, 2 and 6h after blueberry polyphenol intake (766-1791 mg) in healthy men, correlated with plasma concentrations of vanillic acid, homovanillic acid and hippuric acid (29). Ferulic acid has also been shown to restore endothelial function through increasing the bioavailability of NO via a reduction in NADPH-dependent superoxide production in aortas of hypertensive rats (342). Furthermore, chlorogenic acid supplementation to spontaneously hypertensive rats improved endothelium dependent vasodilation with increasing NO bioavailability via NADPH oxidase and decreased blood pressure(343). Certain phenolic acids such as ferulic acid have structural similarities to the pharmacologic NADPH oxidase inhibitor, apocynin and this could explain the mechanistic insights of biological activity of phenolic acid metabolites on NADPH oxidase (344). Inhibitory action of oat phenolic acids on NADPH oxidase activity have been tested in the present study and a reduction at 2h was observed. These results were also paralleled with the appearance of increased concentration of phenolic acids in the blood at 2h, however, there was a reduction with the control intervention as well and the results were non-significant therefore, they were only indicative. Our observations regarding a lack of influence of oat phenolic intake on this enzyme could be related to the dose provided or the variability seen between the study volunteers in terms of absorption of phenolics to the circulation.

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 general population, e.g, women, CVD patients and elderly contributed to the limitations of the trial outcomes. Therefore, the trial was uninformative with respect to the long-term effects that oats consumption might have on CVD biomarkers in general population, and our knowledge, considering also the limited availability of published studies, remains, therefore, incomplete. Further studies are required 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.

In conclusion, our study revealed that a single moderate intake of avenanthramide and phenolic acid enriched oats may improve endothelium dependent and independent microvascular function and this finding is linked with presence of phenolics and their metabolites in the circulation. Oat phenolics may also influence mean % FMD beneficially, because there was a small improvement relative to the control, although this change failed to achieve statistical significance. To our knowledge, this is the first study isolating the phenolic acids together with avenanthramides from soluble fibre and investigating their sole effect on cardiovascular health and when consumed together with other food sources such as coffee and other wholegrains rich in phenolic acids, oat phenolics may have a stronger effect in maintaining the healthy circulatory function and may be contribute to the prevention of cardiovascular disease.

Chapter 4 Chronic vascular effects of oat phenolic acids and avenanthramides in adults with pre- and stage 1 hypertension

4.1 Abstract

Background: Wholegrain consumption is linked to a lower risk of cardiovascular disease. Evidence from randomized controlled trials have established that the consumption of wholegrain oats lowers blood cholesterol, via a mechanism partly mediated by β-glucan soluble fiber. However, oats contain an array of phenolic acids, including ferulic acid and avenanthramides, which may also contribute to the cardiovascular health benefits of oat intake. We investigated whether 4 weeks, daily consumption of oat phenolic acids leads to improvement in markers of CVD risk men and women.

Methods: In a 3 arm crossover double-blind, placebo-controlled trial, 28 volunteers consumed either: 1) oatmeal/oatcake intervention (-containing 48.9mg of phenolic acids and 19.2 mg of avenanthramides); 2) oatbran concentrate+rice porridge/wheat cracker intervention (containing 38.4mg of phenolic acids and 0.5mg of avenanthramides) or 3) rice porridge/wheat cracker intervention (containing 13.8 mg of phenolic acids). All treatments were matched in macro- and micronutrients, energy (500kcal) and total soluble fiber (4.8g). The primary endpoint was flow mediated dilatation (FMD) and other cardiovascular endpoints were laser Doppler imaging (LDI), 24h ambulatory blood pressure (AMBP), pulse wave analysis (PWA), LDL/HDL cholesterol, platelets and endothelial cell-derived extracellular vesicles (EVs). All measures were taken at baseline and after three, 4 week long intervention periods and two washout periods.

Results: Consumption of high phenolic oats for 4 weeks led to a significant improvement in 24-hour SBP (-1.16 mm Hg), night time SBP (-5.1 mm Hg) and night time DBP (-2.3 mm Hg) ($P<0.05$, $P<0.01$ and $P<0.05$, respectively). Our data indicates an increase by 1.09 % ± 0.41 % in FMD response following high phenolic oat intake between baseline and post-intervention. However this difference did not reach to significance. Small increases in endothelial-dependent and –independent microvascular function and small decreases in total and LDL cholesterol after the consumption of moderate and high phenolic oat intervention did not achieve significance. The number of endothelial and platelet derived EVs remained unchanged after the consumption of any of the interventions.

Conclusion: The findings of this study provide additional evidence regarding the role wholegrain oat intake may play in a healthy diet. We provide evidence that the phenolic components within oats may underpin a part of their beneficial actions in cardiovascular protection.

4.2 Introduction

High intake of wholegrains has been associated with reduced incidence and mortality from coronary heart disease, stroke , type-2 diabetes and certain cancer types, as reported by a recent systematic review and meta-analyses of prospective studies and randomized controlled trials(281). Meta-analyses of human intervention studies focusing on health benefits of wholegrain oats reported that regular consumption of these cereals can lower blood cholesterol(271, 345), and improve postprandial glycaemic control and insulin sensitivity(275). Oats are a source of proteins, unsaturated fatty acids, vitamins, minerals (282), dietary fibre including soluble β-glucan and phenolic compounds (phenolic acids and avenanthramides) (21). Of the numerous published epidemiological and human intervention studies concerning the health effects of oats, the overwhelming focus to date has been on the benefits of increased wholegrain oat consumption and on diets or interventions incorporating β-glucan. However, the roles and contributions of bioactive phenolic acids and avenanthramides to health benefits of oats are still not known.

Findings from previous studies suggest that polyphenols present in fruits, vegetables and whole grains may beneficially impact cardiovascular health (162, 346). The ability of these compounds or their circulating metabolites to attenuate well characterized, medically significant CVD risk factors such as hypertension (194, 195, 347), abnormal lipid metabolism (195, 348) and endothelial dysfunction(203, 349-351) have been reported by well-designed chronic randomized controlled trials, and mixed findings are also observed on measures of arterial stiffness improving with high flavonoid food/beverages such as cocoa flavanols (210). In contrast, oats contain predominantly phenolic acids, most notably hydroxybenzoic acids and hydroxycinnamic acids(21, 115) . Hydroxybenzoic acids include protocatechuic, syringic, vanillic , *p*-hydroxybenzoic and gallic acids, while hydroxycinnamic acids are ferulic, *p*-coumaric, *o*-coumaric, caffeic and sinapic acids(115). These compounds are concentrated in the outer layers of the oat grain in three forms : 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 cell wall polysaccharides (dietary fibre and lignin)(289). In addition, oats are unique, with respect to diet, in containing a structurally similar group of phenolic acids known as avenanthramides. These soluble acids are esters of 5-hydroxyanthranilic acid with p-coumaric (2p aka A), and ferulic (2f aka B), and caffeic (2c aka C) acids (290, 291).

There are a number of human intervention trials showing that circulating phenolic acids are positively correlated with improvements in measures of vascular function including flow mediated dilation (FMD) and laser doppler imaging (LDI) after the consumption of cranberries(283), blueberries(352), champagne(31) and coffee(30). Additionally, oat avenanthramides were also shown to be bioavailable in humans and promote cardiovascular physiology in *in vitro* studies (119, 152, 165, 337). Therefore,

oat phenolics may also mediate similar cardiovascular health benefits in humans. A limited number of randomized controlled trials have investigated the effects of oat consumption on vascular function and blood pressure with reporting mixed findings. Increases in % FMD by 1.80± 0.50% have been reported in elderly subjects (>60yrs) after consumption of wild green oat extract (1500mg/day) for 12 weeks(280). In contrast, in a study of oat and wheat based bread interventions, showed no changes in FMD were found in hypercholesteraemic patients after a 6 weeks of consumption (279). Studies of mildly hypertensive subjects showed significant 6mm Hg reduction in SBP (353)and of 7.5 mm Hg SBP and 5.5 mm Hg DBP (354) over a 6-week oat interventions. It has also been reported that ambulatory 24h BP was not significantly altered in hypertensive men following oat intake for 12 weeks (355). Although some of these studies report the amount of fibre given to the subjects, the nutritional content of oats in particularly the amounts of phenolics (phenolic acids and avenanthramides) provided were not reported. Therefore, it is difficult to evaluate the potential impact of oat phenolic intake or synergistic effect of phenolics and fibre on vascular function.

There have been many mechanisms proposed for this potential to improve human vascular function. Most importantly, the ability of polyphenol rich foods to attenuate endothelial dysfunction with increasing NO bioavailability has been reported by numerous human intervention studies (22, 31, 188, 189, 192). NO is a vasodilator playing crucial role in the vascular homeostasis, any defect in its production or activity may lead to endothelial dysfunction, an early stage of atherosclerosis(187, 356). On the other hand extracellular vesicles (EVs) are small membrane derived vesicles originate from different cell types such as platelets and endothelial cells as a result of cell activation or apoptosis (90). Research suggest that circulating EVs increase in relation to CVD risk factors such as hypertension(103), endothelial dysfunction (100, 357) abnormal lipid profile(358), smoking(359) and type -2 diabetes(97) and they may be involved in disease pathogenesis. While the evidence is very limited, there are few studies examining the effects of polyphenols in particularly cocoa flavanols on the number of circulating endothelial and platelet derived EVs and endothelial functional integrity (99, 220-222).

This randomized controlled trial aimed to address the chronic effects (i.e. after 4 weeks) of daily consumption of whole grain oats, with particular consideration given for the levels of phenolics (avenanthramide and phenolic acids) they contain, on vascular function and circulating EVs in pre- or stage1 hypertensive adults.

4.3 Materials and Methods

4.3.1 Subjects

Volunteers were recruited from the University of Reading and surrounding area using the Hugh Sinclair Unit of Human Nutrition database, local media and local GP practices. Eligible volunteers were required to be in general good health, but with moderately high blood pressure (i.e. systolic 120 - 159 mmHg and diastolic 75 - 99 mmHg). The inclusion criteria were non-smoking men and women, women taking the contraceptive pill or on hormone replacement therapy and aged 27 - 75 y. The exclusion criteria were: abnormal biochemical/haematological results assessed at the health screening, hypertension (i.e. SBP/DBP ≥ 160/100 mm Hg), BMI >35 kg/m^2, current smoker or ex-smoker ceased <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, anti-inflammatory, antibiotic or antidepressant medication, allergies to whole grains, dairy and/or lactose intolerance, parallel participation in another research project, had the flu vaccination or antibiotics treatment within 3 months of the 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 or, if of reproductive age, were not using a reliable form of contraception. Of the 87 volunteers screened, 36 were recruited and randomized into the study, with 28 participants completed the study, 13 women and 15 men. The reasons given by the 8 participants who dropped out were: medical issues (n = 2) or could not commit the time for the visits (n = 6). Consort study flow diagram is represented in **Figure 4.1.**

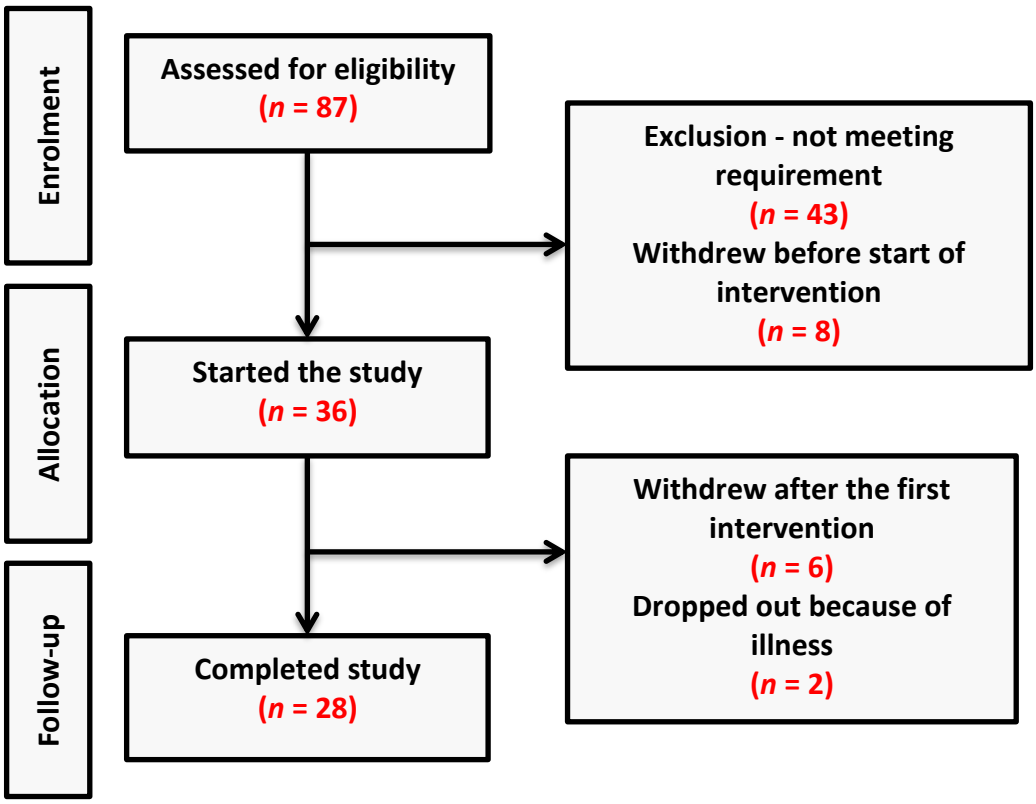

Figure 4.1 CONSORT study flow diagram

4.3.2 Study Design

A three-arm, double-blinded, placebo-controlled randomised crossover intervention study was conducted where volunteers were required to attend the Hugh Sinclair Unit of Human Nutrition. The primary outcome of the study was flow-mediated dilatation of the brachial artery (FMD). Secondary outcome measures were microvascular endothelial function (measured by Laser Doppler Iontophoresis), 24 h ambulatory blood pressure (AMBP) ,arterial stiffness (Pulse Wave Analysis, PWA) , enumeration of circulating extracellular vesicles and blood lipid levels which are all markers associated with CVD risk.

During a 2 week run-in period and the total study duration, volunteers were asked to completely refrain from eating oats (apart from the study interventions), taking dietary, probiotic or prebiotic supplements, drink no more than 400ml/d of tea and only polyphenol-low coffee (i.e. a highly roasted commercial instant coffee provided by the researchers at the Hugh Sinclair Unit, University of Reading), maintain their habitual dietary and activity patterns and keep their body weight within 1kg of their starting weight. The study lasted 20 weeks and consisted of 3 periods: subjects consumed either different doses of the intervention foods, or an energy matched control intervention, selected in

random order for 4 weeks, followed by 4-week washout period (without consuming any treatment products) and then switched to the next randomised assigned treatment. The study included six visits: baseline for each treatment (visit 1, 3 and 5) and at the end of the 4-week treatment (visits 2, 4 and 6). All visits were standardised: participants attended the Hugh Sinclair Unit of Human Nutrition at the University of Reading after fasting overnight for 12 h (not eating or drinking anything but water). They were instructed to avoid polyphenol containing foods for 24 hours , moderate the consumption of alcohol (≤ 21 units)/week, refrain from exercise 48h prior to the study visits and asked to consume provided standard low fat polyphenol-free meal (<15 g fat) the evening before the visit. Compliance to a 24-hour polyphenol free diet and 12-hour fasting was monitored by a 24-hour dietary recall taken in the morning of each study visit. Adherence to the intake of the intervention materials was monitored using volunteer-maintained intake logs and returned, empty material sachets.

Anthropometrical measures were performed (height, weight and % body fat composition (Tanita BC-418 digital scale; Tanita Europe), following which the volunteers rested for 20 minutes in a supine position in a temperature-controlled (22 ± 1 C), light-dimmed room and blood pressure measurement was taken. FMD, LDI and PWA measurements were 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 and to avoid intake of (poly)phenols, caffeine, alcohol and nitrite/nitrate. A polyphenol-low breakfast (white bread toast, butter, cheese, and water) was provided while the volunteers were in the Hugh Sinclair Unit.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki following a Good Clinical Practice and all procedures involving human subjects were 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). Written informed consent was obtained from all subjects before the study started.

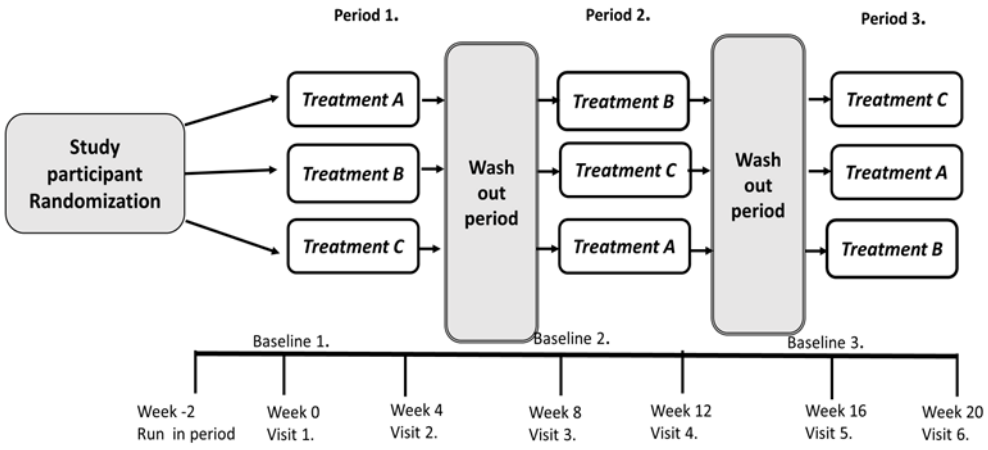

Figure 4.2 Study design.

Timeline of the randomised crossover trial; three 4-week dietary treatments were assessed. The treatments were interspaced by 4-week washout period. Vascular measurements and blood samples were taken during the baseline and at the end of each treatment period.

Table 4.1 Nutritional content and quantities of study products.

Study Products and Content	Control			Moderate Phenolic Oats Intervention			High Phenolic Oats Intervention		
	Breakfast	Afternoon Snack	Daily Total	Breakfast	Afternoon Snack	Daily Total	Breakfast	Afternoon Snack	Daily Total
Study Products (g)									
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(g)									
Energy (kcal)	274.0	226.2	500.2	274.0	226.2	500.2	239.1	261.0	500.1
Carbohydrate	55.04	45.2	101	52.7	45.2	97.9	37.8	30.8	68.7
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
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
β-glucan	0	0	0	4.8	0	4.8	2.7	2.2	4.9
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(mg)	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

Quantities of intervention products phenolic acids, avenanthramides 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.
MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids.

4.3.3 Study intervention Materials

The study volunteers consumed either 1) high dose of avenanthramides and phenolic acids-containing (66.8 g of oatmeal (CDC Dancer oats, provided by PepsiCo, Barrington, USA) and 60 g of oatcake / day) ; 2) moderate dose of avenanthramides and phenolic acids-containing (17 g of oatwell blend with 63.3 g cream of rice (B&G Foods, Inc) and 60g cream crackers) or 3) control diet (68.8 g cream of rice mixed with 8.1 g of cellulose and 4.8 g of pectin and 60 g cream crackers). To achieve the β-glucan matched moderate dose of avenanthramides and phenolic acids oat treatment we used Oatwell, an oat bran concentrate product, containing 28% β-glucan (**Table 4.1**).

All interventions were measured by using HPLC as previously described in Chapter 2 (320) for phenolic acid and avenanthramide content and analysed for macro and micronutrients by Campden BRI (Gloucestershire, UK). All dry materials were frozen until needed to prevent degradation. Both interventions were well tolerated by all subjects, and no adverse events were reported. The breakfast products were packaged and labelled by PepsiCo and Covance Ltd (Ithaca, USA) 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.

4.3.4 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 interventions followed by control intervention or vice versa). Study Product Intake Logs were completed upon the dispensation of the study product to each subject.

4.3.5 Assessment of vascular function and Twenty-four-hour AMBP

Assessment of vascular function was carried out with FMD and LDI as previously described Chapter 3.

Arterial stiffness of peripheral vessels was measured in triplicate with the use of a radial pulse wave analysis (SphygmoCor; AtCor Medical) by a single researcher. BP was measured with an automatic oscillometric digital BP monitor (OMRON) before the measurement and a sensitive transducer was used to record the applanation tonometry of the radial artery to detect waveform traces of the peripheral waveform. The corresponding aortic waveform was obtained using a validated transfer

function (209, 360) and mean arterial pressure, pulse pressure (PP), heart rate (HR), and augmentation index (AIx) were calculated. AIx was corrected for an HR of 75 beats/min (%).

Twenty four hour AMBP was measured with the use of A/A-grade automated oscillometric AMBP monitors (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 taken following vascular measurements and breakfast over a 24 h period. Twenty four-hour AMBP and heart rate were recorded every 30 min from 07:00 h to 21:59 h and every 60 min from 22:00 h to 6:59 h, the two initial measurements were discarded. Average 24-h day and night time measurements were analysed with the use of sleep times. PP was expressed as the difference between SBP and DBP. Participants recorded activities and sleep hours throughout the measurement period in a diary.

4.3.6 Diet diary analysis

For diet diary analysis, all the food and drinks consumed by volunteers, for four consecutive days, one during the weekend and the other three during the week was recorded before each pre-intervention visit (1, 3 and 5). 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.

4.3.7 Biochemical analysis

Fasted venous blood samples were collected in serum separation tubes (VACUETTE, Greiner Bio-One) and allowed to stand for 30 min before centrifugation (1700 x g for 15 min at 20°C). Lipids (total cholesterol (TC), LDL cholesterol, HDL cholesterol and triacylglycerol (TAG) and glucose were quantified from serum and analysed with ILAB 600 chemistry analyser by using enzyme-based colorimetric tests (ILAB600; Werfen (UK) Ltd.; reagents and analyser: Instrumentation Laboratory Ltd) to determine the baseline characteristics of the study population. Lipids (total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol) were also assessed at every visit. LDL cholesterol was estimated by means of the Friedewald formula, i.e. ((LDL= (TC –HDL) – (TAG)/2.2)) (361). Screening samples were frozen at -20 °C and study visit samples were kept in -80 °C until analysis.

4.3.8 Enumeration of extracellullar vesicles

Fasted venous blood was collected into sodium citrate tubes (BD vacutainer), the first tube was discarded, and the remainder was processed <30mins of collection. Samples were centrifuged at 1500 X g for 15 min at 20°C and then at 13000 X g for 2 min at 20°C to obtain platelet poor plasma (PFP). PFP samples were kept at −80 °C until analysis. PFP (5 µl) was incubated with FcR (5 µl) (Miltenyi Biotec, Bergisch Gladbach, Germany) + argatroban (1 µl) (Sigma-Aldrich, St. Louis, Missouri, US) for 15mins at room temperature, in the dark to provide calcium and facilitate the binding of Annexin V (AV) to PS on EVs. PFP was then stained with 3 different antibodies; 2 µl AV (Fisher Scientific, Massachusetts, US) to identify PS positive EVs, 2 µl CD41-PE (Diagnostica Stago, New Jersey, US) to identify platelet derived PS positive EVs (PDEVs) and 4 µl CD105-eF450(Life Technologies , Invitrogen, California, US) to identify vascular endothelial EVs(EDEVs). Fluorescence-minus-one (FMO) for each antibody including isotype matched controls (1 µl of Immunoglobulin eF450 (IgGeF450) (Life Technologies, Invitrogen, California, US) and 2 µl IgG-PE (Diagnostica Stago, New Jersey, US) were also performed with same amount of PFP and FcR+argatroban. AV buffer alone (Cambridge Bioscience, Cambridge, UK) and PFP alone with the addition of FcR+argatroban were also included in the protocol to check for any background contamination. AV buffer or phosphate-buffered saline (for AV FMO) (Sigma-Aldrich, St. Louis, Missouri, US) were then added to all test tubes to make up a final volume of 50 µl and samples were incubated for 15 mins at room temperature, in the dark. Prior to flow cytometry analysis, samples were further diluted with 200 µl of AV buffer or phosphate-buffered saline (for Annexin V FMO) and analysed on a BD FACSCanto II flow cytometer (BD Biosciences, New Jersey, US) with a medium flow rate. Flow cytometer was set with using a Cytometer Setup and Tracking (CS&T) beads (BD Biosciences, New Jersey, US) to perform quality control of the instrument's optics, electronics and fluidics, and for adjusting fluorescence compensation. Rainbow calibration particles (8 peak beads) (BD Biosciences, New Jersey, US) with eight different fluorescent intensities were also used to calibrate and track the performance of flow cytometer. EV gate was set with using ApogeeMix beads (Apogee flow systems, Nortwood, UK) containing aqueous mixture of silica beads with different diameters 180nm, 240nm, 300nm,590nm,880nm and 1300nm and a refractive index of 1.43 which is similar to the refractive index of biological vesicles. The EVs gate was set with using light scattering between 0.3µm and 1µm to include the events above the electronic noise of the cytometer and to cover upper size threshold for EVs (**Figure 4.3**). 10,000 events were recorded from EVs gate with using fluorescence channel signals, samples were triggered with Allophycocyanin (APC) channel to detect EVs labelled with AV and CD41 and triggered with Pacific Blue channel to detect EVs labelled with CD105eF450. From these events, staining for AV showed the PS-exposing, PS^+ EVs (**Figure 4.4- A and B**) and staining for CD41 showed platelet derived PS^+CD41^+ double positive EVs (**Figure 4.4- C and D**). Additionally, endothelial derived EVs were demonstrated as PS^+CD105^+ (**Figure 4.4- E and F**). BD Trucount tubes (BD Biosciences,

New Jersey, US) were used to determine the absolute counts of EVs/ml. Values were reported as counts per ml of blood.

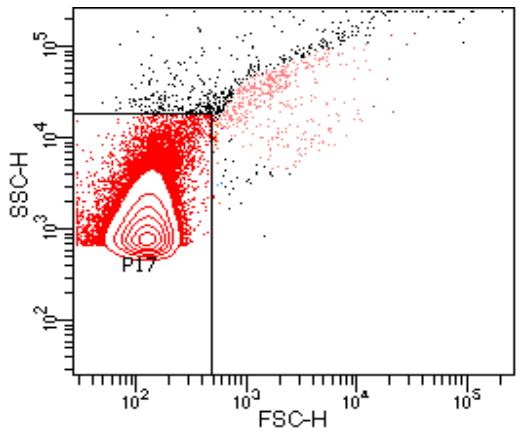

Figure 4.3 Gating strategy for EV enumeration with flow cytometry.

A total of 10,000 events were acquired in gate P17 to allow the analysis of EVs. Light scatter threshold based on EV granularity and size was used to set the gate. The gate was set to include events between 0.3 µm (lower detection limit defined as a threshold above the electronic noise of the flow cytometer) and 1 µm (which is the upper size threshold for EVs). SSC, side scatter and FSC, forward scatter.

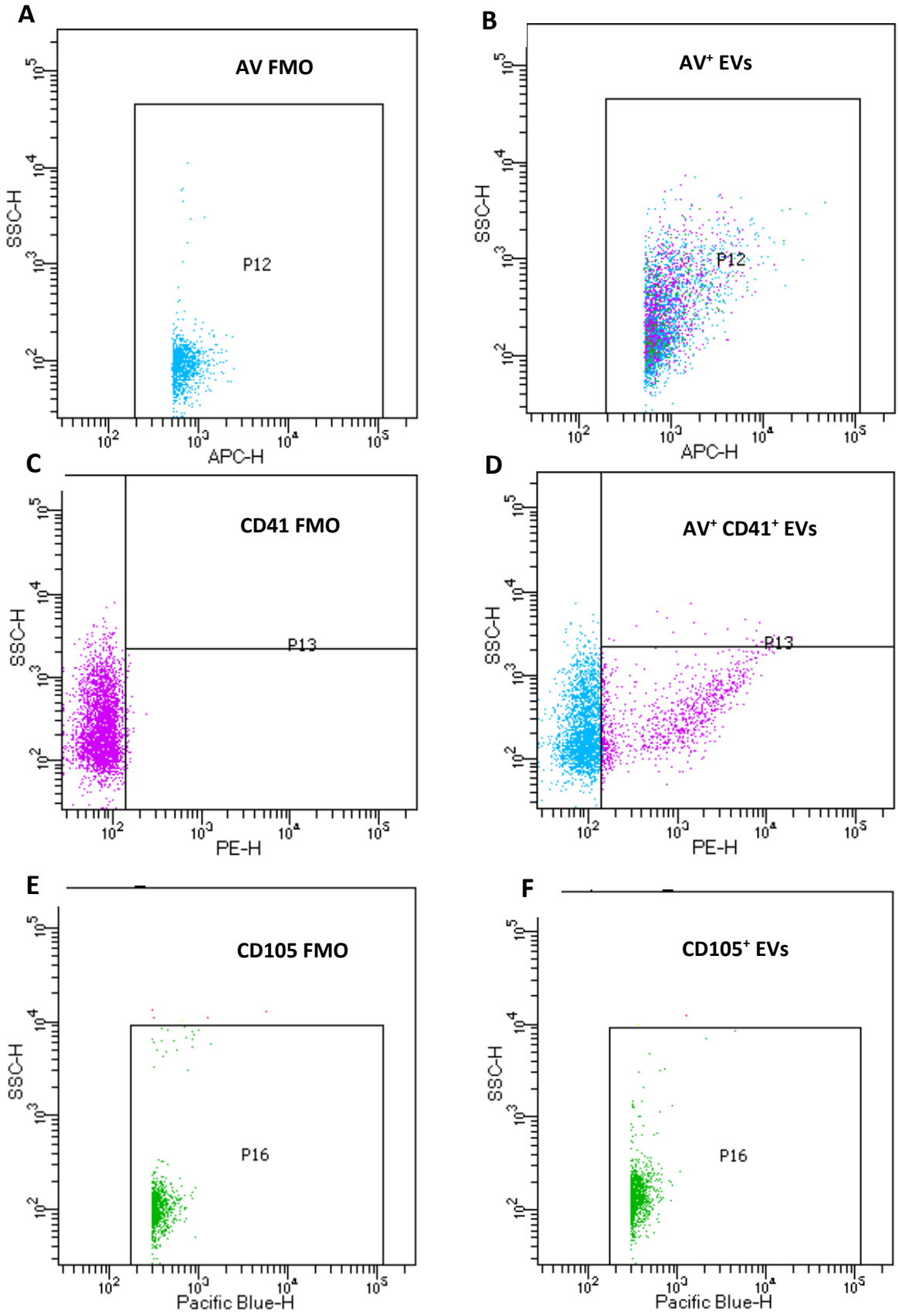

Figure 4.4 Analysis of EVs in PFP with flow cytometry after staining with AV, CD41 and CD105.

FMO controls for each antibody is shown to demonstrate any other events in the absence of the antibody. **A**: shows the FMO for AV triggered in APC and **B**: shows the AV^{+}EVs. **C**: shows FMO for CD41 and **D**: shows AV^{+} $CD41^{+}$ double positive platelet derived EVs (PDEVs). **E**: shows FMO for CD105 triggered in Pacific Blue and F: shows $PS^{+}CD1O5^{+}$ endothelium derived EVs (EDEVs).

4.3.9 Sample Size and Statistical Analysis

The power calculation was made for the primary clinical outcome measure (FMD) 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 subjects. 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 and circulating levels of EVs), PROC MIXED in SAS 9.4 was used to fit a linear mixed model with fixed effects for a 3 period crossover design and participant as a random effect. A significance level of 5% was adopted. Where the repeated measures model showed significant differences, pairwise comparisons between the interventions were performed using *post hoc* analysis (Bonferroni adjustment). The graphs were generated with Graphpad version 8 and data presented in the text, tables, and figures represent the means and SEMs.

4.4 Results

4.4.1 Baseline characteristics of study population and tolerance of intervention

Baseline characteristics of 28 pre-hypertensive and stage 1 hypertensive, mixed gender individuals were measured. (**Table 4.2**). The intervention and control products were well tolerated by all subjects and no adverse events were reported.

Table 4.2 Baseline clinical characteristics of the study population at screening

Baseline Characteristics	Mean ± SEM
Age (y)	49.6 ± 2.3
BMI (kg/m^2)	26.7 ± 0.7
Systolic blood pressure (mm Hg)	129.7 ± 1.9
Diastolic blood pressure (mm Hg)	80.1 ± 1.2
Heart rate (bpm)	71.1 ± 2.3
Plasma glucose (mmol/L)	5.6 ± 0.1
Plasma total cholesterol (mmol/L)	5.3 ± 0.1
Haemoglobin (g/L)	145.5±2.1

BMI – Body Mass Index

4.4.2 Twenty-four Hour Ambulatory Blood Pressure (AMBP)

There were statistically significant mean changes in 24 SBP ($p<0.05$) and night time SBP ($p<0.01$) and DBP ($p<0.05$) following the intake of high phenolic oat relative to moderate phenolic or control interventions in relation to baseline measurements (**Figure 4.5** and **Table 4.3**). A significant decrease was observed in 24 SBP following the intake of high phenolic oats (126.23± 1.53 mm Hg) relative to baseline (127.39± 1.67 mm Hg) over the timeframe of the intervention. Significant reductions in night time SBP and DBP were also observed following the consumption of high phenolic oat intervention (110.58 ± 2.05 mm Hg and 66.42 ± 1.51 mm Hg, respectively), compared to baseline (115.68 ± 1.68 mm Hg and 68.68 ± 1.12 mm Hg, respectively), a mean difference of -5.1 mm Hg and - 2.3 mm Hg, respectively.

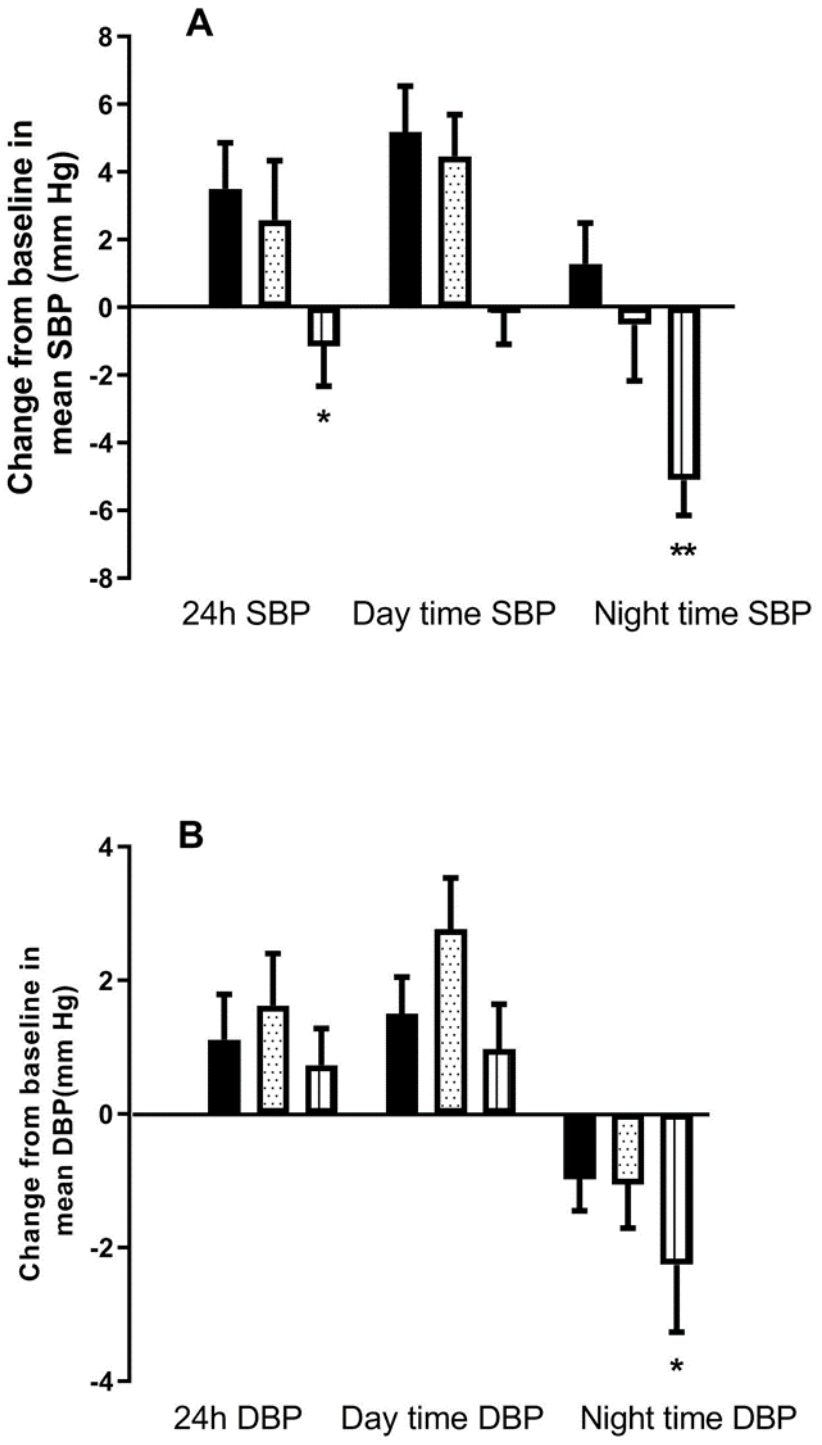

Figure 4.5 Changes in 24 hour, day-time and night-time systolic and diastolic ambulatory blood pressure .

Changes from baseline in 24 hour, day-time and night-time systolic (**A**) and diastolic (**B**) ambulatory blood pressure (BP) (mm Hg) measured at baseline and following to 4 week consumption of control (filled bar), moderate (dotted bar) or high phenolic (lined bar) oats **.** Values are expressed as means ± SEMs from Table 4.3. A linear mixed model analysis fixed effects of 3 period crossover design and participant as a random effect was performed, n = 28, followed by Bonferroni 's correction to adjust for multiple testing. *p<0.05, **p<0.01 and *** p<0.001.

Table 4.3 Twenty-four hour ambulatory blood pressure measurements of participants at baseline and following to 4 week consumption of control, moderate or high dose phenolics[a].

Blood Pressure Measure (mm Hg)	Control		Moderate Phenolic Oats Intervention		High Phenolic Oats Intervention		p-value
	Baseline	Post	Baseline	Post	Baseline	Post	
24 h SBP	125.8 ± 1.8	129.3 ± 2.4	126.2 ± 2.3	128.8 ± 2.8	127.4 ± 1.7	126.2 ± 1.5*	0.050
Day SBP	129.7 ± 1.9	134.9 ± 2.4	129.5 ± 2.2	134.0 ± 3.0	131.7 ± 1.8	131.6 ± 1.9	0.234
Night SBP	113.2 ± 2.2	114.5 ± 2.3	114.2 ± 2.7	113.7 ± 2.4	115.7 ± 1.7	110.6 ± 2.1**	0.011
24 h DBP	76.8 ± 1.2	77.9 ± 1.3	77.5± 1.3	79.1 ± 1.5	77.7 ± 0.9	78.4 ± 0.9	0.816
Day DBP	79.9 ± 1.1	81.5 ± 1.3	80.4 ± 1.4	83.2 ± 1.6	80.9 ± 0.9	81.9 ± 1.2	0.676
Night DBP	68.4 ± 1.7	67.5 ± 1.3	68.8 ± 1.6	67.8 ± 1.5	68.7 ± 1.1	66.4 ± 1.5*	0.054

Twenty-four-hour ambulatory blood pressure (n=28), systolic blood pressure (SBP) and diastolic blood pressure (DBP). [a] Values are presented as mean measurement ± SEM. Data were analysed using linear mixed model with fixed effects of 3 period crossover design and participant as a random effect followed by Bonferroni's correction to adjust for multiple testing , *p<0.05, **p<0.01 and *** p<0.001.

4.4.3 Vascular function

There was no significant changes in endothelium dependent brachial artery vasodilation, measured by FMD following consumption of control, moderate- or high phenolic oat interventions when compared to baseline. There was, however non-significant, dose-dependent increases from baseline measurements by 0.43, 0.50 and 1.09 % respectively (**Figure 4.6,** calculated from **Table 4.4**) after 28-days of chronic consumption (p=0.91).

Similarly, microvascular reactivity, assessed by LDI was not significantly different following the consumption of control, moderate- or high phenolic oat interventions in relation to baseline (**Figure 4.7** and **Table 4.4**). However, there were, small but measurable increases following the consumption of high phenolic oat intake, AUC (146.6 PU/h^{-1}) and iAUC (43.7 PU/h^{-1}) of endothelium-dependent vasodilation, facilitated by Ach (p=0.96 and p=0.76 , respectively). Non-significant increases were also found following the high phenolic oat intake, AUC (150.6 PU/h^{-1}) and iAUC (380.1 PU/h^{-1}), and moderate phenolic oat intake, AUC(285 PU/h^{-1}) and iAUC (552.1 PU/h^{-1}) of endothelium-independent vasodilation facilitated by SNP (p=0.31 and p=0.5, respectively).

No significant changes were found for arterial stiffness measured by PWA and expressed as AIx (%) and AIxHR75 (%), following either treatments (p=0.32 and p=0.81, respectively) (**Table 4.4**).

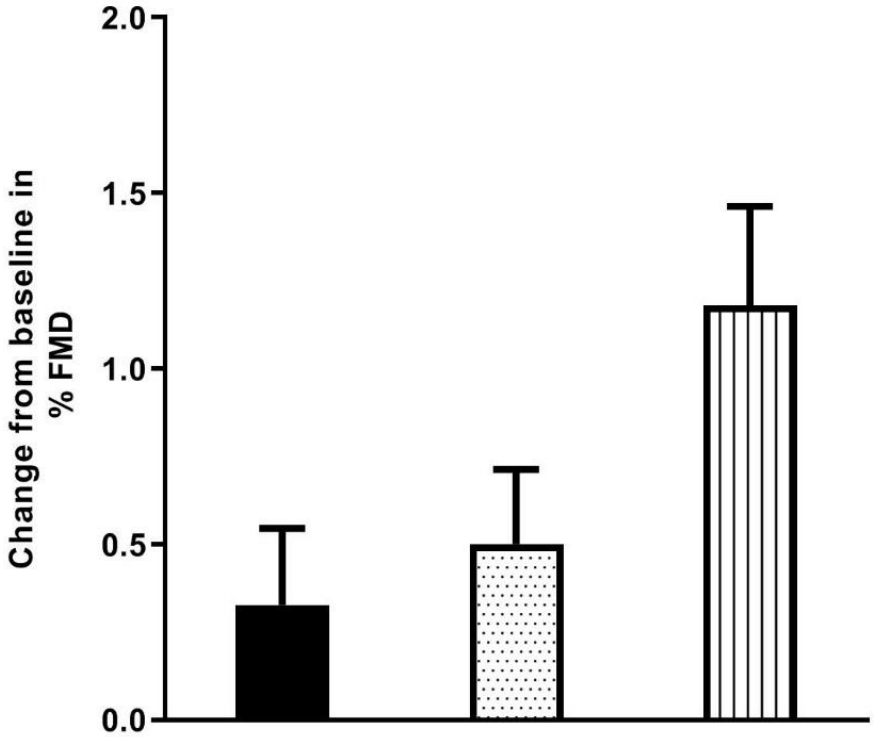

Figure 4.6 Changes in FMD.

Changes in % FMD from baseline after 4 week consumption of control (filled bar), moderate (dotted bar) or high phenolic (lined bar) oats. Values are expressed as means ± SEMs, n=28 from **Table 4.4**. Data were analysed using linear mixed model with fixed effects of 3 period crossover design and participant as a random effect followed by Bonferroni's correction to adjust for multiple testing where there was a significant finding.

Table 4.4 Vascular measures of participants at baseline and following to 4 week post-consumption of control, moderate and high phenolic oats [a].

Vascular Reactivity Measure	Control		Moderate Phenolic Oats Intervention		High Phenolic Oats Intervention		
	Baseline	Post	Baseline	Post	Baseline	Post	p-value
FMD (%)	5.7 ± 0.47	6.1 ± 0.5	5.6 ± 0.4	6.1 ± 0.4	5.8 ± 0.4	6.9 ± 0.4	0.911
LDI-AchAUC	1188.7 ± 233.2	1156.5 ± 233.4	1312.8 ± 171.5	1290.9 ± 236	1569.6 ± 227.1	1716.3 ± 292.9	0.967
LDI-SNP AUC	1171.8 ± 200.8	1233.3 ± 150.7	1127.8 ± 127.3	1412.8 ± 256.8	1459.4 ± 241.6	2011.5 ± 386	0.314
LDI-Ach iAUC	320.7 ± 163.4	298.2 ± 137.6	378.1 ± 135.6	344.5 ± 207.2	611.3 ± 220.4	655.0 ± 207.7	0.760
LDI-SNP iAUC	407.2 ± 172.4	353.1 ± 114.4	255.6 ± 65.1	406.2 ± 161.2	492.0 ± 206.3	872.1 ± 286.6	0.500
PWA, AIx (%)	28.0 ± 1.7	28.1±1.7	27.8±2.1	26.8±2.1	27.3±1.8	27.7±1.8	0.325
PWA, AIxHR75 (%)	21.5±1.4	21.4±1.4	21.3±1.8	20.8±1.7	21.2±1.5	20.7±1.5	0.819

Flow-mediated dilatation (FMD) (n=28); Laser Doppler Iontophoresis (LDI); endothelial-dependent vasodilation (Ach) (n=28) and endothelial-independent vasodilation (SNP) (n=28), expressed as both area under curve (AUC) and incremental area under curve (iAUC); Pulse wave analysis (PWA); AIx (n=26); Augmentation index, AIxHR75 (n=25); Augmentation index when corrected for a HR of 75 mm Hg. [a] Values are presented as mean measurement ± SEM, for units see the text. Data were analysed using linear mixed model with fixed effects of 3 period crossover design and participant as a random effect followed by Bonferroni's correction to adjust for multiple testing where there was a significant finding.

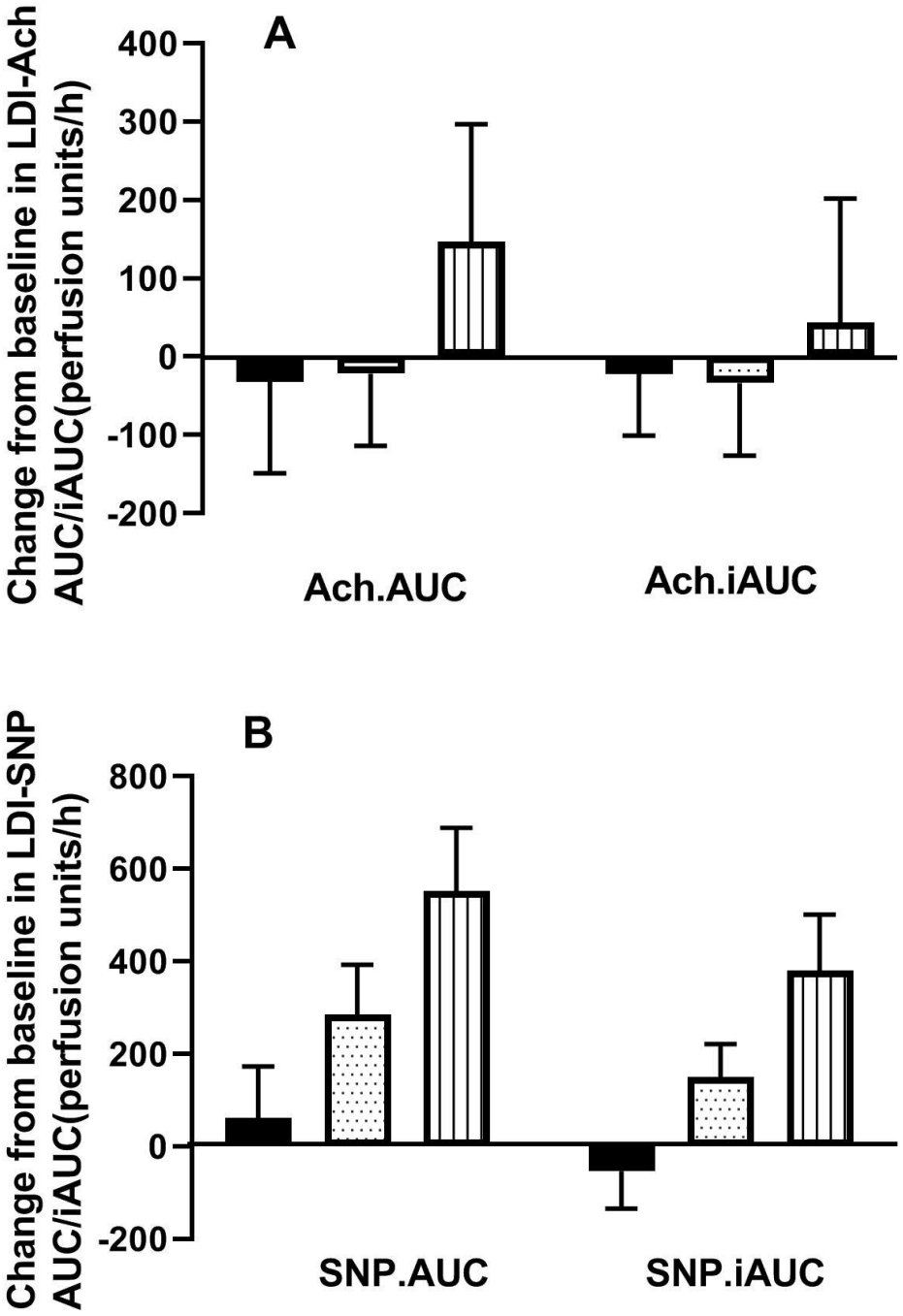

Figure 4.7 Changes in LDI.

Changes from baseline in microvascular function measured with LDI at baseline and following to 4 week consumption of control (filled bar), moderate (dotted bar) or high phenolic (lined bar) oats: (**A**) endothelium-dependent vasodilation (Ach) represented by area under the curve (AUC) and incremental area under the curve (Ach iAUC) (perfusion units per hour, PU/h-1) and (**B**) endothelium-independent vasodilation (SNP), represented by area under the curve (AUC) and incremental area under the curve (SNP iAUC) (perfusion units per hour, PU/h-1).Values are expressed as means ± SEMs from **Table 4.4**. A linear mixed model analysis fixed effects of 3 period crossover design and participant as a random effect was performed, n = 28, followed by Bonferroni 's correction to adjust for multiple testing where there was a significant finding.

4.4.4 Lipid Profile

There were no significant changes in TC (p=0.42), LDL-cholesterol(p=0.24), HDL-cholesterol(p=0.85) and triglycerides(p=0.95) following to consumption of high phenolic, moderate phenolic or control interventions in relation to baseline measurements (**Table 4.5**). However, small, non-significant and therefore only indicative reductions were observed in the following: TC was decreased by -0.2 mmol/L and -0.16 mmol/L in moderate and high phenolic oat interventions respectively between the baseline and post-intervention measurements, compared to a slight increase in the control group. LDL-cholesterol was reduced by -0.07 mmol/L and -0.26 mmol/L respectively, compared to -0.05 mmol/L in the control. HDL-cholesterol also decreased by -0.14 mmol/L and -0.04 mmol/L respectively, compared to -0.02 mmol/L in the control. In contrast, triglycerides increased only slightly by 0.17 mmol/L and 0.05 mmol/L respectively, compared to 0.06 mmol/L in the control.

4.4.5 Enumeration of Circulating Extracellular Vesicles

No significant decreases were found in the numbers of circulating EVs analysed with flow cytometry as PS^+ EVs (p=0.19), PS^+ $CD41^+$, PDEVs (p=0.77) and PS^+ $CD105^+$, EDEVs (p=0.07) following to consumption of high, moderate phenolic or control interventions (**Figure 4.8** and **Table 4.5**). Although, there was a very slight reduction observed in PS^+ EVs following the consumption of high or moderate phenolic oats relative to baseline measurements, this effect did not reflect to the numbers of PS^+ $CD41^+$, PDEVs and PS^+ $CD105^+$, EDEVs. Additionally, increases in the number of PS^+ $CD105^+$, EDEVs were observed following the consumption of high and moderate phenolic interventions.

Table 4.5 Fasting lipid profile and number of circulating EVs at baseline and following to 4 week consumption of control, moderate or high phenolic oat interventions[a]

Lipids and EVs	Control		Moderate Phenolic Oats Intervention		High Phenolic Oats Intervention		
	Baseline	Post	Baseline	Post	Baseline	Post	p-values
Total cholesterol (mmol/L)	5.2 ± 0.2	5.3 ± 0.2	5.4 ± 0.2	5.2 ± 0.2	5.2 ± 0.2	5.1 ± 0.2	0.426
LDL cholesterol (mmol/L)	3.2 ± 0.2	3.1 ± 0.2	2.9 ± 0.3	2.9 ± 0.2	3.2 ± 0.1	2.9 ± 0.2	0.241
HDL cholesterol (mmol/L)	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.5± 0.1	1.5 ± 0.1	1.5 ± 0.1	0.849
Triglycerides (mmol/L)	1.2 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.953
PS^+ EVs	$2.7x10^7±2.7x10^6$	$2.9x10^7±5.7x10^6$	$2.7 x10^7±2.6x10^6$	$2.6 x10^7±2.4x10^6$	$2.9 x10^7±2.7x10^6$	$2.8x10^7±4x10^6$	0.195
PS^+ $CD41^+$ PDEVs	$1.5x10^7±1.7x10^6$	$1.3x10^7±1.3x10^6$	$1.4 x10^7±1.5x10^6$	$1.4 x10^7±1.5x10^6$	$1.5x10^7±1.2x10^6$	$1.5x10^7±1.5x10^6$	0.776
PS^+ $CD105^+$ EDEVs	$8.3x10^6±1.7x10^6$	$7.6x10^6±1.7x10^6$	$8.3x10^6±1.8x10^6$	$8.9x10^6±1.9x10^6$	$7.3x10^6±1.2x10^6$	$8.9x10^6±1.6x10^6$	0.074

Fasting lipid profile (n=28) measured as total cholesterol (mmol/L), low-density lipoprotein (LDL)-cholesterol (mmol/L), high density lipoprotein (HDL)-cholesterol (mmol/L) and triglycerides (mmol/L). Extracellular vesicles (EVs) (n=23) measured with flow cytometry as phosphatidylserine positive EVs (PS^+ EVs), phosphatidylserine and CD41 double positive platelet derived EVs (PS^+CD41^+PDEVs), phosphatidylserine and CD105 double positive endothelial derived EVs (PS^+ $CD105^+$ EDEVs). [a] Values are presented as mean measurement ± SEM. Data were analysed using linear mixed model with fixed effects of 3 period crossover design and participant as a random effect followed by Bonferroni's correction to adjust for multiple testing where there was a significant finding.

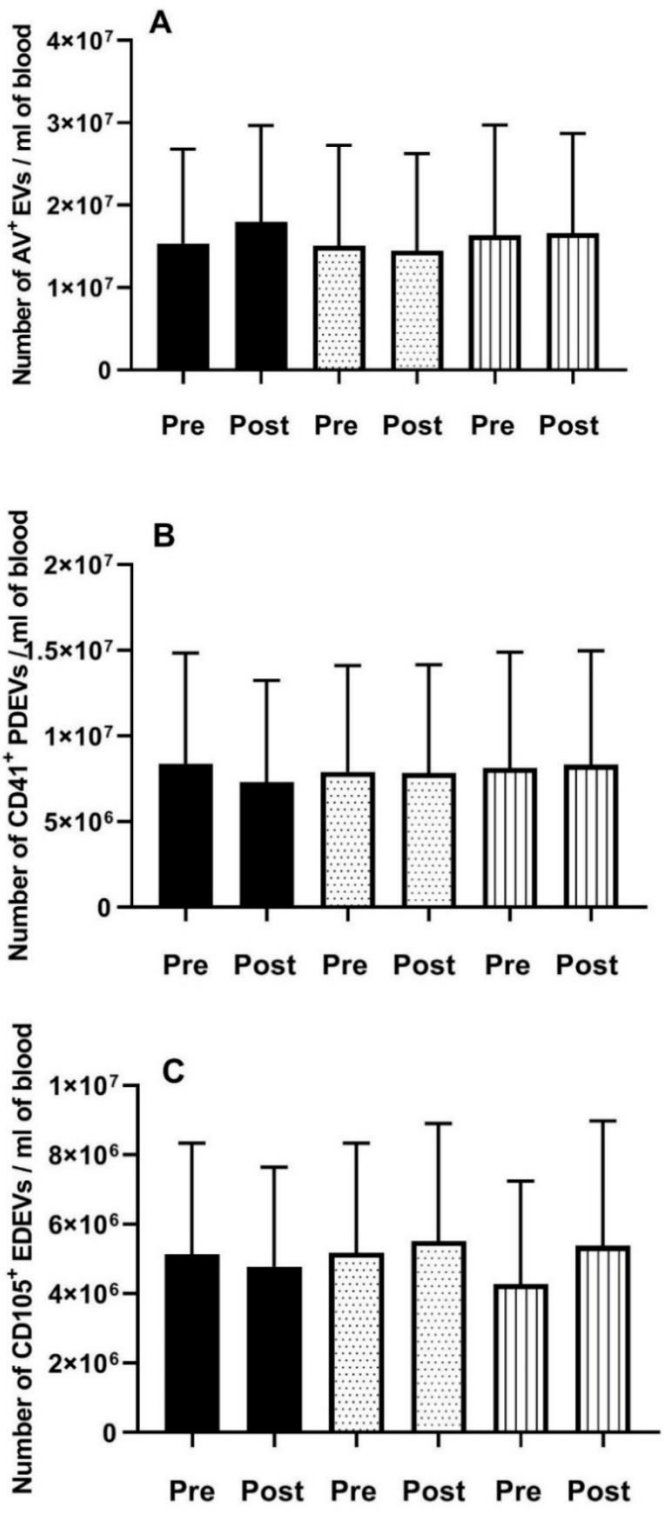

Figure 4.8 Changes in circulating EVs.

Effects of control (black bars), moderate (dotted bars) or high (lined bars) phenolics oat intervention on numbers of circulating EVs measured by flow cytometry pre and post treatment: (**A**) PS^+ EVs , (**B**) PS^+ $CD41^+$ PDEVs , (**C**) PS^+ $CD105^+$ EDEVs. Values are expressed as means ± SEMs from **Table 4.5.** A linear mixed model analysis fixed effects of 3 period crossover design and participant as a random effect was performed, n = 23, followed by Bonferroni 's correction to adjust for multiple testing where there was a significant finding.

4.4.6 Nutrient Intake Summary

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

Table 4.6 Daily consumption of the principal dietary nutrients is shown for the control, moderate and high phenolic oats interventions[a].

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

MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids. [a]All values are means ± SEMs, n = 28.

4.5 Discussion

In this chronic intervention study, we examined the vasoactive potential of the intake of moderate and high phenolic containing oats in pre or stage 1 hypertensive adults, over a 4-week intake period, relative to iso-caloric low phenolic containing control. Our results indicate that the consumption of oats at our highest dosage of phenolic acids (48.8 mg phenolic acids and 19.3 mg avenanthramides) significantly reduced 24 h SBP ($p<0.05$), night time SBP ($p<0.01$) and DBP ($p<0.05$) relative to baseline and in comparison to moderate phenolic containing oats 38.4mg of phenolic acids and 0.5 mg of avenanthramides) and control (13.8 mg phenolic acids). Similarly, although not significant, consumption of high or moderate containing phenolic oats also improved FMD of the brachial artery and microvascular blood.

Measurement of 24h ambulatory blood pressure demonstrated significant decreases in 24h SBP (-1.2 mm Hg), night time SBP (-5.1 mm Hg) and DBP (-2.3 mm Hg) following the consumption of high phenolic oats relative to baseline measurements and moderate phenolics or control interventions. No changes were observed in day time BP following any of the interventions, which may be due to the confounding impact of daily activity levels (362). In agreement with our findings, consumption of whole grain oats in a similar pre or stage 1 hypertensive study group showed a significant 6 mm Hg reduction in SBP after a 6-week intervention (353) and reductions of 7.5 mm Hg SBP and 5.5 DBP following wholegrain oats consumption in hypertensive subjects (354). It must be noted that these studies used single blood pressure measurements, which is considered inferior to 24h-BP assessment in providing robust data (363). Conversely to our study, it has been reported that ambulatory 24-h BP was not significantly changed in hypertensive men following oat intake over a 12-week period (355). This inconsistency is reflected across other studies with polyphenol interventions, with long-term flavanol-rich cocoa (> 2 weeks, 500-700mg of flavanols) and tea (< 6 months, 58-500mg polyphenols) consumption found to reduce both SBP and DBP by 2 mmHg and 1-3 mmHg, respectively (194, 195), similarly studies with anthocyanin-rich foods such as berries resulted in reduced BP in overweight or hypertensive men (199, 200). With respect to other phenolic acid rich foods, studies testing coffee consumption reported no changes in blood pressure following 1 week of intake (400 mg chlorogenic acids), whereas acute consumption of coffee with the same dose showed -2.4 and -1.4 mm Hg reduction in SBP and DBP, respectively in healthy individuals (202, 364). Chronic consumption of phenolic rich olive leaf extract were also reported to lower day time (-3.9 mmHg) and 24h-SBP, (-3.3 mmHg) and day time(-3 mmHg) and 24h- DBP(-2.42 mmHg) in pre-hypertensive men(365). While the previous evidence is inconsistent, our results provide further evidence that oats high in phenolics may reduce BP, particularly night time BP which is reported to be a better predictor of cardiovascular events (366, 367). Importantly, -5.1 mmHg and -2.3 mmHg decrease seen in our night time SBP and DBP can be considered as medically relevant improvements as observational studies suggest that a 2 mm Hg decrease in both SBP and DBP

are associated with 6% and 7% decreases in CHD risk and 10% and 15% decreases in heart attack and stroke, respectively(368, 369).

Our primary outcome, FMD, showed small, but non-significant improvements: 0.43, 0.50 and 1.09 % in response to control, moderate and high phenolic oats interventions, respectively. Previous data focusing on the effects of wholegrain oat intake on vascular health has predominantly focussed on the impact of fibre, in particular β-glucan, and have not been controlled for phenolic content (276-280). Despite this, in agreement with our data, studies testing the vascular effects of oat based interventions in healthy subjects for 4 or 6 weeks, also report no significant improvements in endothelial function (276, 277) and this is also true in hypercholesterolemic patients over a similar timeframe (278, 279). In contrast, significant increases in % FMD (1.80 ± 0.50%) have been reported following the consumption of a wild green oat extract (1500 mg/day) for 12 weeks in healthy older adults (280). Wild green oat extract was reported to be rich in bioactives such as avenanthramides, saponin, vitexin and isovitexin but this study did not analyse the nutritional content of the intervention product(280). With respect to other polyphenol-containing foods/beverages, there is extensive evidence to suggest foods rich in flavanols, for example cocoa (370) and tea(349) might be effective to improve vascular function. A meta-analysis of chronic cocoa/chocolate trials, reported improvements in FMD by 1.45 %, on average, at flavanol intake levels of 100-200 mg/day (371). Other studies testing a variety of polyphenol rich foods reported mixed results, for example a study using wild blueberry drink providing 375 mg of anthocyanins and 127 mg of chlorogenic acid for 6 weeks did not show any improvements in FMD in subjects with CVD risk factors (218). However, olive oil polyphenols (30mg/day for 8 weeks) have been reported to benefit vascular system with improving endothelial function and increasing NO levels in pre-hypertensive women (203). Although these studies were not examining the vascular benefits of phenolic acids similar to those present in oats, it is known that the metabolism of flavonoids in the colon leads to the formation of phenolic acids which have structurally similarity to those found in oats, and it may be these same components that underpin the vascular activity of such flavonoid interventions in humans (22).

Consistent with our findings, a study providing flavonoid-rich fruit and vegetable puree drink for 6 weeks (296 mg anthocyanins per day) showed non-significant improvements in endothelial dependent microvascular function in healthy subjects(372). However, another study similarly investigating flavonoid rich fruit and vegetable intake for 6 weeks reported significant increase in endothelial dependent micro vascular function in healthy men (373). In the current study, no changes were found in arterial stiffness determined by PWA and represented as AIx and AIx@75 following the consumption of either treatments. Existing data related to oat consumption and arterial stiffness is limited, an acute study reportedly failed to show any changes in AIx@75 following the consumption of 60 g oatmeal(374). Furthermore, long term studies focusing on polyphenols and arterial stiffness showed inconsistent findings with most promising evidence coming from consumption of cocoa flavanols and

cranberry polyphenols showing a positive effect (212, 375). Taken together, despite the non-significance of our findings on FMD, LDI and PWA and individual measures being relatively variable in our trial population making statistical significance hard to achieve, our results tentatively show that the intake of oats with highest phenolics tends to benefit the vascular system the most.

No significant changes in blood lipid profile including total (TC), LDL and HDL cholesterol and triglycerides were found in this current study following any of the interventions. While there is a well-established effect of oat consumption on TC and LDL cholesterol mostly in hypercholesteraemic subjects (25, 345), our results are consistent with some other studies which have concluded that oat or polyphenol consumption led to no significant difference in plasma lipid profiles of healthy, overweight or pre hypertensive subjects (201, 376-378). However, it must be noted that 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. Circulating EVs as emerging CVD risk factors majorly expose phosphatidylserine (PS) as a sign of early cell activation process and EV shedding (379-383), in this current study, we tested the effect of chronic phenolic rich oat consumption on the number of these PS+ circulating vesicles in pre or stage 1 hypertensive subjects. Our results did not show any significant differences in the numbers of PS+EVs, PS+ platelet derived extracellular vesicles (PDEVs) and PS+ endothelial derived extracellular vesicles (EDEVs) following the consumption of high phenolic, moderate phenolic oats and control. While there is no evidence regarding to the effect of wholegrains or oat consumption on the number of circulating EVs, there are few studies examining the effect of polyphenols, particularly cocoa flavanols on this outcome and reporting reductions in the numbers of PDEVs and EDEVs (99, 219, 221, 222). Different phenotyping techniques and gating strategies between these studies and our study need to be considered when comparing the findings. Moreover, the threshold of the flow cytometer was set as >300nm to detect EVs in the current study whereas some of the previous studies reported threshold values as high as < 3µm which indicates the possibility of reporting platelets and apoptotic bodies as EVs as well (219, 222). While the existing evidence shows that cocoa flavanols might reduce the number of circulating EVs particularly, EDEVs and these results are shown to be paralleled with improvements in FMD and circulating nitric oxide species suggesting that flavanols may improve endothelial integrity in subjects with CVD(99), our study showed that oat phenolics do not affect the numbers of circulating EVs. This could be because of oat phenolics not being as biologically active as flavanols on EVs or the dose provided in the current study, which was at a realistic dietary intake level, was not sufficient to induce an effect.

A number of limitations of the current study are worth noting. The relatively short duration, along with the sample size, which was too small to have enough power to draw conclusions across all the outcomes tested, are likely the principal limitations. Although we reported significant changes in blood pressure following the intake of high phenolic oats, our study was powered for the primary outcome, FMD, therefore, we are cautious with our conclusions. Lack of compliance with the intervention diet

can be another potential factor influencing our lack of statistically relevant findings. We used strict controls over dietary intake of oats but, as with the majority of dietary intervention studies, we relied on self-reporting of diet by individuals. Our diet dairy analysis of the study participants showed no significant differences in the dietary intake of carbohydrate and fat (384), fibre (385) and MUFA (386, 387), or any other dietary components that could influence specific outcomes such as cholesterol and lipid profiles. However, it must be noted that our high phenolic intervention contained higher levels of PUFA and MUFA when compared to moderate phenolics and control. This could be considered as a limitation of our study as intake of MUFA and PUFA may also beneficially influence the blood pressure and vascular function (362, 388). Finally, the findings from the study population, which consisted of pre or stage-1 hypertensive adults, cannot be extended to the general population, and further work is necessary to observe whether, for example, elderly or CVD patients experience similar benefits. Therefore, further studies with more sample size, and for a longer intervention period are necessary for the effects to become better resolved and statistically significant.

In conclusion, the present study has strengthened the existing body of evidence that oat consumption may beneficially influence the cardiovascular health. Our data suggest that at normal dietary intake levels, chronic consumption of oats may reduce blood pressure at a medically relevant levels and beneficially effect human vascular system. These effects may be mediated, in part, by phenolic acids and avenanthramides contained within oats. Additional long-term studies are required to examine potential mechanisms of action behind these findings and to understand the exact contribution of phenolic acids and avenanthramides and their metabolites to vascular benefits of oats.

Chapter 5

5.1 General Discussion

CVD accounts for one third of the mortality in the UK and a diet rich in vegetable, fruit and wholegrains may help to maintain a healthy cardiovascular function (3, 10). Wholegrain intake is associated with reduced risk of CVD and dietary fibre content of cereals has been reported to play an important role in cardiovascular health (15, 281). Evidence suggest that oats are effective in lowering blood cholesterol and improving post-prandial glycaemia, via a mechanism partly related to their high β-glucan soluble fibre (25, 275). Oats are also a rich source of phenolic acids and avenanthramides, although the degree to which these compounds contribute to cardiovascular health benefits of these cereals has not been investigated in humans (115). Recent dietary interventions using phenolic acid-rich foods/beverages (blueberry, coffee and champagne) are capable of improving vascular function (29-31). These studies reported improvements in microvascular blood flow and large artery function which shown to be driven by small phenolic acids including ferulic acid, caffeic acid and p-coumaric acid with mainly modulating endothelial function by increasing NO bioavailability. Oats which may promote healthy cardiovascular aging, have a similar phenolic acid profile and are sustainable, relatively cheap product that offers an alternative to more expensive and less sustainable foods/beverages such as blueberries and champagne.
Therefore, main scope of this thesis was to investigate whether consumption of phenolic acids and avenanthramides found within oat-based diets may promote vascular function particularly via their potential to enhance NO bioavailability to the vascular endothelium in an acute and chronic manner in humans. The main findings of this thesis include;

- Phenolic acid and avenanthramide concentrations and compositions of commercial oat products are broadly similar and oats are an important source of dietary phenolics to the consumer providing 15.8-25.1 mg in a portion.
- Consumption of 50 mg of phenolics resulted in acute improvements in microvascular function and correlated with circulating phenolic acid metabolites in blood in pre- and stage 1 hypertensive men. Large variability observed in phenolic absorption between study volunteers.
- Daily consumption of an oat based diet for 4 weeks providing 68.1 mg of phenolics significantly decreased 24 hour SBP, night time SBP and DBP and resulted in non –significant, dose-dependent changes in microvascular and vascular function in adults with pre- and stage 1 hypertension.

Initially, phenolic acid and avenanthramide composition and amounts of a variety of commercial oats including oat bran, oat bran concentrate, rolled, flaked oats and oat cakes were aimed to be determined. Analysis of commercial oat products showed that fibre rich oat bran concentrate has the highest total concentrations of phenolics whereas other oat products contained lower but similar amounts. In agreement with previous studies, bound fraction of phenolic acids formed the majority of the total content whereas free+conjugated fraction were lower and similar in all products including oat bran concentrate(295, 296). High amounts of bound phenolic acids quantified in this study demonstrated the importance of gut microbiota that may assist the release of colonic phenolic acid metabolites to enhance the health benefits of oats (149). On the other hand, similar amounts of more bioaccesible free+conjugated fraction of phenolic acids and avenanthramides which may be absorbed from the small intestine showed that potential health benefits could be delivered by all products analyzed regardless of type (333). Based on our sample range that included the most available products to the consumer, we concluded that oats, a popular breakfast cereal in the UK can provide 15.8-25.1 mg of phenolic acids and avenanthramides in a 40g portion whereas oat bran concentrate provides 16.7 mg in 11 g portion. This analysis mainly provided data about the average dietary intake of phenolics from oats and informed our following clinical work to use a realistic dose to test the vascular effects of these compounds in humans.

Secondly, an acute randomized controlled trial was performed to test the ability of oat phenolic acids and avenanthramides to produce improvements in circulatory function over 24 h in pre and stage 1 hypertensive men using established, gold standard, clinical measures of blood flow in the body. Our findings suggested small non-significant improvements in vascular function, measured by FMD, at 1h and 24h following to a single intake of oats (90g, containing 50mg of total phenolics) relative to macro- , micronutrient and fibre matched cream of rice control. Lack of effect observed in our FMD results broadly disagrees with other acute human intervention trials tested higher doses of flavonoid and/or phenolic acid containing foods/beverages such as cocoa, blueberries, cranberries and coffee and reported changes as early as 1 h after consumption(29, 30, 283, 389). As a proposed underlying mechanism, inhibitory effects of oat phenolics on NADPH oxidase activity was tested but no changes were observed in this current acute trial whereas a blueberry dose response study providing >766mg of polyphenols reported improvements in FMD and also correlated phenolic acids including vanillic acid, homovanillic acid and hippuric acid with a decreased NADPH oxidase activity between 1- 2h and 6h (29).

On the other hand, we reported significant changes in endothelium dependent and independent microvascular function at 2h following the consumption of oats. These results were in agreement with a study that showed improvements in endothelium independent microvascular function at 4h and 8h with higher total excretion levels of phenolic acids following the consumption of

champagne(31). Changes in endothelial independent microvascular function in our study were positively correlated with blood concentrations of phenolic acid metabolites including ferulic acid sulfate, ferulic acid glucuronide and p-coumaric acid. However, we observed large variations in phenolic acid absorption between study participant's, 2 h after intake, some participants were classified as absorbers with a greater change in microvascular response and others low/non absorbers with small or no improvements of microvascular function. In agreement with the variability of microvascular response we observed, a very recent review systematically analyzing the data from published studies stated that there are number of factors such as individual's gut microbiota composition and function, genetic make- up, age, sex, ethnicity, BMI and pathophysiological status which could be responsible for variability in absorption, biological responsiveness to polyphenols and these may influence the cardiometabolic response (390).

Finally, a chronic human study was conducted to test the effect of high and moderate dose of oat phenolic acids and avenanthramides on vascular health. Consumption of high phenolic oats that consisted of 66.8 g of oatmeal and 60 g of oatcake (providing 68.1 mg of phenolics) for 4 weeks led to a significant improvement in 24-hour SBP, night time SBP and night time DBP. In agreement with our findings, few other studies reported a decrease in blood pressure following the consumption of oats in pre and stage 1 hypertensive men (353, 354). While these studies did not distinguish the phenolic content of oats and mainly attributed the observed effects to oat based diets or fibre content of oats, our study provides a clearer evidence that the decrease observed in blood pressure may also partly be mediated by oat phenolics. Additionally, there is a body of evidence suggests that chronic consumption of polyphenols in various foods may reduce BP. While the studies on cocoa and tea flavanols are being more consistent, there are some studies examining flavanoids in berries and phenolics in coffee and olive oil and showing a reduction in BP (194, 195, 199, 202, 364, 365). It must be noted that structurally bigger polyphenols such as flavonoids can be metabolized into smaller phenolics which may exert health benefits in humans, therefore findings of these studies might be relevant to our findings. We also observed dose dependent changes in vascular, microvascular response and LDL cholesterol following the consumption of high and moderate dose of phenolics, however these results were not significant and high variability of responsiveness was observed between the participants. There is a valuable amount of evidence that shows the potential beneficial effects of polyphenol rich foods including cocoa , tea ,berries and olive oil on endothelial function whereas oat intake was generally shown to fail to induce any improvements in FMD but extensively reported to reduce total cholesterol and LDL-cholesterol , particularly in subjects with hypercholesterolemia (22, 25, 203, 277-279, 349). Because of these conflicting findings between our study and others, also the dose-response improvement we observed in our measures, we suggest

that a bigger sample size is necessary to understand the long-term effects of oat phenolics on vascular and microvascular function and cholesterol.

In summary, our results suggest that acute consumption of structurally smaller oat phenolic acids and avenanthramides at provided doses might be effective on microvascular function whereas higher doses of phenolic acids or consumption of these compounds together with other classes of polyphenols such as flavonoids might be required to exert biological actions on vascular function. We can suggest that free+conjugated phenolics that are likely to be absorbed from small intestine after consumption might be responsible for the significant effect that we observed in microvascular function at 2h. Inter-individual variability that we observed in phenolic absorption shows that every individual may not equally benefit from these compounds and this should be considered in future strategies of personalized nutrition for a better prevention of CVDs. In comparison to the acute study, our chronic study demonstrated stronger evidence that the intake of oat phenolics may reduce blood pressure that are in terms of magnitude, possibly medically relevant. Variability observed in the measures of chronic study further supported the variability to the responsiveness to the intervention, although we did not measure phenolic acid metabolite levels chronically to define this. Overall, this study adds further evidence that consumption of oats, containing phenolic acids and avenanthramides may help to maintain a healthy cardiovascular system.

5.2 Future Perspectives

In this thesis, we addressed a number of research questions while highlighting some gaps for further studies. We showed that the acute improvement in microvascular function might be mediated by phenolic acid metabolites quantified in blood. However, our chronic study lacked similar type of pharmacokinetic analysis. Therefore, further studies are required to measure the blood and urinary levels of phenolic acid metabolites in order to understand the accumulation over long-term intake. Based on a previous bioavailability study performed in our group, we showed elevated total urinary excretion levels of phenolics between 0-2h and 4-8h following the consumption of oatbran (See Appendix). Therefore, we could suggest that a collection time of blood and urine samples in a chronic study could start from 8h after consumption and extend beyond this time point to examine whether bound phenolics are absorbed to the bloodstream and also to see if phenolics accumulate over time to exert beneficial effects on vascular function. On the other hand, variabilities in individual's gut microbiota composition may limit the action of colonic metabolites, therefore, enzymatic treatments of oat products to release the bound phenolics into free forms to increase the bio accessible phenolic content might be help to increase health benefits of oats. Together with circulating metabolites, in order to examine the mechanisms of action, the inhibitory effects of oat phenolics on NADPH oxidase

activity and circulating levels of NO might be measured in a chronic manner to understand the effects of phenolic acid exposure on cells following repeated ingestion.

Finally, conducting similar types of studies for longer intervention periods, in other populations including elderly or people with other cardiovascular disease factors may help to better define the effects of oat phenolics on vascular system.

Appendix 1: Excretion of avenanthramides, phenolic acids and their major metabolites following intake of oat bran

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Mol Nutr Food Res: doi,10.1002/mnfr.201700499

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Abbreviations: ND, not detected; sMRM, scheduled multiple reaction monitoring

Keywords: avenanthramide, absorption, metabolism, phenolic acid, oat bran, wholegrain

Abstract

Scope: Wholegrain has been associated with reduced chronic disease mortality, with oat intake particularly notable for lowering blood cholesterol and glycemia. To better understand the complex nutrient profile of oats, we studied urinary excretion of phenolic acids and avenanthramides after ingestion of oat bran in humans.

Methods and results: After a 2d (poly)phenol-low diet, 7 healthy men provided urine 12h before, and 48h after, consuming 60g oat bran (7.8µmol avenanthramides, 139.2µmol phenolic acids) or a phenolic-low (traces of phenolics) control in a crossover design. Analysis by UPLC-MS/MS showed that oat bran intake resulted in an elevation in urinary excretion of 30 phenolics relative to the control, suggesting that they are oat bran-derived. Mean excretion levels were elevated between 0-2 and 4-8h, following oat bran intake, and amounted to a total of 33.7±7.3µmol total excretion (mean recovery: 22.9±5.0%), relative to control. The predominant metabolites included : vanillic acid, 4- and 3-hydroxyhippuric acids and sulfate-conjugates of benzoic and ferulic acids, which accounted collectively for two thirds of total excretion.

Conclusion: Oat bran phenolics follow a relatively rapid urinary excretion, with 30 metabolites excreted within 8h of intake. These levels of excretion suggest that bound phenolics are, in part, rapidly released by the microbiota.

Introduction

Increasing the daily intake of wholegrain cereals by 90g has been associated with reduction in mortality from cardiovascular disease by 27%, total cancer by 15%, respiratory disease by 22%, diabetes by 51% and infectious diseases by 26%, as indicated by recent meta-analysis of 45 prospective studies (15). Human intervention studies have to date largely focused on wholegrain oats (*Avena sativa* L.), with meta-analyses establishing that regular oat intake lowers blood cholesterol (391, 392) and improves insulin sensitivity and post-prandial glycemic control (275). Although oats only account for 1% of world grain production, they, unlike more widely consumed grains, are almost exclusively consumed as wholegrains and therefore a rich dietary source of high quality proteins, minerals, vitamins, soluble β-glucan fiber and phenolic compounds (i.e. phenolic acids and avenanthramides), all of which are concentrated in the outer bran layers (393).

β-glucan is, at least in part, responsible for the health benefits of oats (275, 391, 392), while there is limited *in vitro* evidence that avenanthramides (394) and phenolic acids (28) may also promote beneficial cardiovascular physiology. Phenolic acid intake in Europe is on average 605 mg/d, with the main dietary sources being coffee (75%), fruits (5.6%) and wholegrain products (5.5%) (395). Wholegrain is the richest dietary source of ferulic acid, which has a mean intake of 38 mg/d in Europe (395), although a number of other phenolic acids and phenolic alkaloids, notably the avananthramides, are present in oats, either in the 'free form', as soluble conjugates, or as insoluble bound forms (including ester-linked to fiber) (396). Understanding the urinary excretion (an indirect measure of absorption) of oat phenolic compounds following the dietary intake of whole oats or oat bran is a key prerequisite for determining which phenolic metabolites may mediate the health benefits of oats.

Previous data using oat phenolic extracts or wheat have indicated that phenolics transfer to the circulation following intake (123, 150), whilst limited data exist for the urinary excretion of phenolics and other bioactive components from whole oats or oat bran at a dietary level. Notably, the intake of 150 mg of avananthramides (highly concentrated oat extract), led to detection of avenanthramides 2c, 2f and 2p at nanomolar concentrations in plasma between 0.25 and 5h, peaking at 2h (123). While this study did not examine metabolism, data from studies in animals suggest that avenanthramide 2c is metabolized to avenanthramide 2f, dihydroavenanthramide 2f and 2c, four hydroxycinnamic acids, and 5-hydroxyanthranilic acid following oral intake (313). Furthermore, intake of 94 g wholegrain wheat bread, containing 87 mg of ferulic acid or aleurone-enriched white bread led to the appearance of ferulic acid-sulfate, dihydroferulic-sulfate, hippuric acid and two hydroxyhippuric acids in plasma, with the former two reaching peak plasma concentrations of 84 nM and 9 nM at 1 and 7 h after intake, respectively (150), along with 12 other phenolic acid metabolites in 48h urine, suggesting that ferulic acid is subject to extensive metabolism and that some fiber-linked ferulic acid is released later during transit through the gastrointestinal track through action of the microbiota (150).

The aim of our study was to examine the urinary excretion of avenanthramides and phenolic acids following intake of 60g oat bran. Specifically, the study focused on three objectives; 1) to establish peak urinary excretion intervals of phenolic acids and avenanthramides; 2) to determine the temporal nature of oat phenolic release from the bound state in the gastrointestinal tract; and 3), to identify and quantify the range of phenolic metabolites derived from the oat phenolics using comprehensive UPLC-MS/MS methods.

Materials and Methods

Chemicals and reagents

Avenanthramide 2c, avenanthramide 2f, avenanthramide 2p, 2,4-dihydroxy benzoic acid, *p*-coumaric acid, caffeic acid, isoferulic acid, syringic acid, salicylic acid, o-coumaric acid, vanillic acid, syringaldehyde, ferulic acid, sinapic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, protocatechuic acid, isovanillic acid, gallic acid, homovanillic acid, hippuric acid, dihydroferulic acid, dihydrocaffeic acid, 4-hydroxyphenylacetic acid and 2-hydroxyhippuric acid were obtained from Sigma-Aldrich (see Supporting Information Table 1 for IUPAC names). Dihydroferulic acid-4-*O*-glucuronide, isoferulic acid-3-*O*-sulfate, ferulic acid-4-*O*-glucuronide, dihydroxybenzoic acid-3-*O*-glucuronide and 5-hydroxyanthranilic acid were obtained from Toronto Research Chemicals Inc. 4-hydroxyhippuric acid and 3-hydroxyhippuric acid were purchased from Enamine (see Supporting Information Table 1 for IUPAC names). All solvents were HPLC grade and were obtained from Sigma-Aldrich or Fisher Scientific. While we acknowledge that avenanthramides are phenolic alkaloids, the terms phenolics is used throughout the paper to include both avenanthramides and phenolic acids.

Extraction and analysis of oat bran phenolics

Soluble and bound phenolic fractions were extracted from oats using an established method (294), with addition of hexane defatting steps adapted from (397) and preservation of phenolics during alkali hydrolysis using ascorbate and EDTA adapted from (398). Phenolic acids and avenanthramides were separated using an Agilent 1100 series HPLC equipped with a Kinetex biphenyl column (100 Å 250x4.6 mm, 5μm; Phenomenex) with a Security Guard ultra biphenyl cartridge (Phenomenex). Sample injection volume was 20 μL, the flow rate 1 mL/min and mobile phases consisted of 0.1% formic acid (v/v) in water (solvent A) and 0.1% formic acid (v/v) in methanol (solvent B). The solvent gradient consisted of 5% B at 0 min, 25% B at 20 min, 26% B at 25 min, 35% B at 30 min, 36% B at 40 min, 70% B at 53 min, 95% B at 56 min, 95% B at 61 min, 5% B at 62 min, 5% B at 65 min. The absorbance was recorded at 254, 280 and 320nm and quantification was based on 12 point linear calibration curves

(mean R^2 > 0.994) and as a ratio to the internal standard (i.e. 3,5-dichloro-4-hydroxybenzoic acid) to account for losses during extraction.

Study design

Seven healthy men aged 25-62 years were recruited from the local community. Exclusion criteria were as follows: recent (last 3 months) use of antibiotics, flu vaccination or dietary supplements. All participants gave written informed consent prior to study commencement and the study was performed at the Hugh Sinclair Unit of Human Nutrition, University of Reading (UK) between July 2014 and September 2014. The study was approved by the University of Reading Research Ethics Committee (Reference number: 31/15), followed the principles of the Declaration of Helsinki and was registered on ClinicalTrials.gov under NCT02574039.

The study was designed as a non-blinded, randomized, controlled trial, where participants attended two experimental visits that were identical with the exception of the two study meals: 1) Test intervention consisting of 60 g oat bran porridge, 200 ml semi-skimmed milk and 100 ml water, microwaved (2min); or 2) a control consisting of 100g white bread, 14g butter, golden syrup, one boiled egg and 200 ml semi-skimmed milk; given in random order. Participants arrived at the Nutrition Unit at 8am or 9am to consume the study meal within 10 min. Urine was collected at 11 specific intervals relative to study meal consumption: -12h to 0h (i.e. baseline), 0h to 2h, 2h to 4h, 4h to 6h, 6h to 8h, 8h to 12h, 12h to 24h, 24h to 28h, 28h to 32h, 32h to 36h and 36h to 48h (see Figure 1). Collected urine was kept on ice packs, the excretion volume measured and aliquots were stored at -80°C with and without 5% formic acid acidification. During the 48h urine collection period, participants consumed a low-phenolic meals provided to them (lunch: a white bread cheese sandwich and toffee yoghurt; dinner: macaroni cheese pasta, white bread roll and crème brulée; breakfast: white toast with butter and golden syrup, a boiled egg and glass of milk), whilst drinking water *ad libitum*. Prior to each experimental visit, participants followed a diet low in (poly)phenols for 48h (i.e. no fruits, vegetables, wholegrains, pulses, spices, herbs, nuts, seeds, chocolate, tea and coffee), and attended an overnight fast after having consumed a low-(poly)phenol dinner (a cheese pasta bake, white bread roll and crème brulée). Compliance to the dietary restrictions was assessed using food intake diaries and questionnaires. One volunteer was excluded from the final dataset, due to non-compliance to the dietary restrictions (i.e. volunteer reported intake of (poly)phenol-rich foods and baseline and post-control urine contained high levels of phenolics).

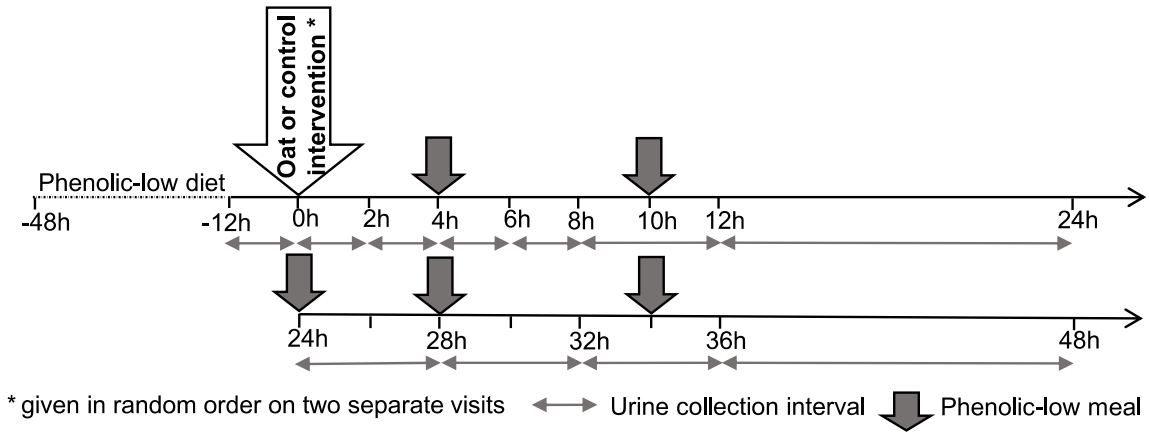

Figure 1: Study Design Overview.

Solid phase extraction

Phenolic acids were extracted from urine using a validated method (399) with minor modifications. Briefly, 1 mL of urine was spiked with an internal standard (i.e. 3,5-dichloro-4-hydroxybenzoic acid) and subsequently extracted using solid phase extraction cartridges (Strata-X columns 500mg/6mL; Phenomenex). These were washed with 12 mL of 0.1/99.9 v/v hydrochloric acid/water, dried for 30 min under vacuum, soaked in 0.1/99.9 v/v hydrochloric acid/methanol for 10 min and eluted into glass vials with 7 mL 0.1/99.9 v/v hydrochloric acid/methanol. Samples were evaporated to complete dryness under speedvac at room temperature. The dried samples were resuspended in 250 µL of mobile phase (0.1/5/94.9, v/v/v, formic acid/methanol/water) by 30s vortexing, 15min ultrasound sonicating and 1h shaking. Samples were stored at -80°C until analysis. For phenolic acids and metabolites the method has a mean ± SD extraction efficiency of 88.3 ± 17.8% as previously reported (399), while we established extraction efficiencies of 102%, 97% and 57% for avenanthramide A, B and C, respectively.

UPLC-MS/MS analysis

The UPLC-electrospray ionisation-MS/MS system consisted of an Aquity UPLC Hclass (Waters) coupled to a Xevo TQ-S micro electrospray ionisation mass spectrometer (Waters) operated using MassLynx software (V4.1, Waters Inc, USA). Compound separation was achieved using an Aquity UPLC HSS T3 1.8µm column (2.1 x 100mm) attached to a Van guard pre-column of the same material and pore size, maintained at 45°C with a flow of 0.65 mL/min and a sample injection volume of 2 µL. The mobile phase consisted of 0.1/99.9 v/v formic acid/water (A) and 0.1/99.9 v/v formic acid/acetonitrile (B); and a mobile phase gradient consisting of: 1% B at 0 min, 1% B at 1 min, 30 % B at 10 min, 95 % B at 12

min, 95% B at 13 min, 1% B at 13.10 min, 1% B at 16 min. A scheduled multiple reaction monitoring (sMRM) method was developed by syringe infusion of 34 analytical standards (see section Chemicals and Reagents and Supporting Information Table 1) to determine sMRM transitions, optimal sMRM modes (i.e. negative or positive) and collision energies (Supporting Information Table 2).

Regarding phase II metabolites, while four authentic standards were used for the sMRM method (i.e. dihydroferulic acid-4-*O*-glucuronide, isoferulic acid-3-*O*-sulfate, ferulic acid-4-*O*-glucuronide and dihydroxybenzoic acid-3-*O*-glucuronide), further putative glucuronide or sulfate conjugated phenolic acid metabolites and feruloyl glycine were added to the sMRM method, even though analytical standards were not commercially available. For these, retention times and sMRM transitions were tentatively identified by injecting a pooled extract of urine (i.e. using urine collected after oat bran intake from all n=7 participants and during all 10 post-intake time intervals). sMRM transitions were taken from the literature (151, 326, 400, 401) or derived from the fragmentation pattern of the phenolic acid aglycones by adding the m/z of glucuronide (i.e. 176) or sulfate (i.e. 80) to the precursor ion and including the appropriate MS/MS fragment. Furthermore, 113 m/z, a common fragment of glucuronic acid, was added as an MS/MS fragment for glucuronide conjugates (326). Collision energies were optimized to a limited extent by injecting the pooled urine extract three times at collision energies -11, -17 and -21 and the best one was selected for each sMRM transition. In the final sMRM, a total of 74 potential compounds were targeted and statistical comparison between the oat and control intervention was used to identify those which are oat bran-derived phenolics. Identification of the phenolic metabolites for which standards were available was based on their retention times and the major sMRM ion transitions of the standards, while the tentative identification of phenolic metabolites for which analytical standards were not available was based on 3-6 sMRM ion transitions (except for benzoic acid-sulfate which only has 2 sMRM ion transitions as reported in the literature (401)) (Supporting Information Table 2).

Quantification was established using the most intense sMRM transition and 11-14 point calibration curves of analytical standards (Supporting Information Table 2). Where pure standards were not available, quantification was conducted relative to standard curves of compounds with similar structures (e.g. the calibration curve of isoferulic acid-3-*O*-sulfate was used to quantify all tentatively identified sulfate metabolites). The limit of detection and quantification were established for each compound as the concentrations of peaks with signal to noise ratios of 3 and 10, respectively, and were lowest for avenanthramide 2p (0.8nM and 2.7nM, respectively) and highest for vanillin or hydroxyphenylacetic acid-*O*-glucuronide (35.0nM and 116.7nM, respectively; Supporting Information Table 2). Blanks and quality controls were run every 10 injections. Validation parameters indicate that the method had good precision and repeatability (inter-day variability: 9.1%; inter-day variability: 13.7%) and good linearity (linear regression coefficient range: 0.990 to 1.000; Supporting Information Table 2)

Sample acidification using 5% formic acid did not significantly affect phenolic compound peak areas (established in n=3 volunteers; data not shown) and therefore non-acidified urine was used for the complete analysis.

Table 1: Content and composition of phenolics in 60g oat bran [a)]

	Fraction		
	Conjugated and free	**Bound**	**% Conjugated and free**
Avenanthramides			
Avenanthramide 2c	0.8mg (2.5µmol)	ND	100%
Avenanthramide 2f	1.0mg (3.1µmol)	ND	100%
Avenanthramide 2p	0.7mg (2.2µmol)	ND	100%
Hydroxycinnamic acids			
Ferulic acid	2.0mg (10.1µmol)	14.8mg (76.4µmol)	12%
p-Coumaric acid	0.4mg (2.3µmol)	3.0mg (18.1µmol)	11%
Caffeic acid	0.1mg (0.4µmol)	1.3mg (7.0µmol)	5%
Sinapic acid	0.9mg (3.8µmol)	1.2mg (5.5µmol)	41%
Hydroxybenzoic acids			
4-Hydroxybenzoic acid	0.2mg (1.8µmol)	0.3mg (1.9µmol)	48%
Vanillic acid	0.4mg (2.4µmol)	0.4mg (2.2µmol)	53%
Syringic acid	0.4mg (2.1µmol)	0.3mg (1.5µmol)	57%
Benzaldehydes			
4-Hydroxybenzaldehyde	0.2mg (1.4µmol)	0.1mg (1.1µmol)	56%
Vanillin	ND	0.2mg (1.3µmol)	0%
Total	7.0mg (32.1µmol)	21.6mg (115.0µmol)	24%

[a)] 60g of oat bran porridge made with 200ml semi-skimmed milk and 100ml water. Heated by microwave. ND, not detected

Statistical analysis

A two-factor repeated-measurement linear mixed model was fitted to analyze hourly urinary excretion data. The model included participants nested within time as a random effect and baseline hourly urinary excretion, intervention, time and interaction as fixed effects. When the model showed a significant interaction effect, *post-hoc* analysis with Tukey-Kramer adjustment was performed. Data are presented as means ± SEMs. P-values of <0.05 were considered statistically significant, and statistical analysis was performed by using R programming language version 3.1.2 (R Development Core Team, 2014).

Results

Phenolic composition of the oat bran intervention

Twelve phenolic compounds were detected in the oat bran used for the trial, with eight phenolic acids being present in both soluble and bound fractions, three avenanthramides only present in the soluble fraction and vanillin only in the bound fraction (Table 1). The intervention diet (60g oat bran) contained 28.6mg total phenolics (24% in the soluble fraction), with ferulic acid being the predominant phenolic acid (16.8mg) followed by *p*-coumaric acid (3.3mg) and the three avenanthramides collectively amounting to 2.5mg. Although the control meal and all other meals consumed during the 48h study periods were not analyzed, they consisted of white wheat bread and pasta and would therefore be expected to contain only low amounts of phenolic acids (402).

Identification of oat bran-derived phenolic compounds in urine

A wide range of phenolic compounds were detected in urine at baseline and after the control meal intake (Supporting Information Table 3). However, the hourly excretion of 30 individual phenolic compounds was elevated following intake of oat bran compared to the control intervention (significant mixed model P-value of intervention and/or time*intervention; Supporting Information Table 3), suggesting that they are derived from the ingested oat bran. These phenolics were identified as avenanthramides 2f and 29 phenolic acids (nine aglycones, four glycine conjugates, ten sulfate conjugates and seven glucuronide conjugates; Supporting Information Table 2). Excretion of hippuric acid, the dominant phenolic acid in human urine, was not statistically higher after the oat bran intervention relative to the control and was thus not identified as an oat bran-derived metabolites (mixed model time*intervention interaction P-values: 0.36; Supporting Information Table 4). In total,

44 targeted phenolic compounds were statistically similar between interventions (Supporting Information Table 4).

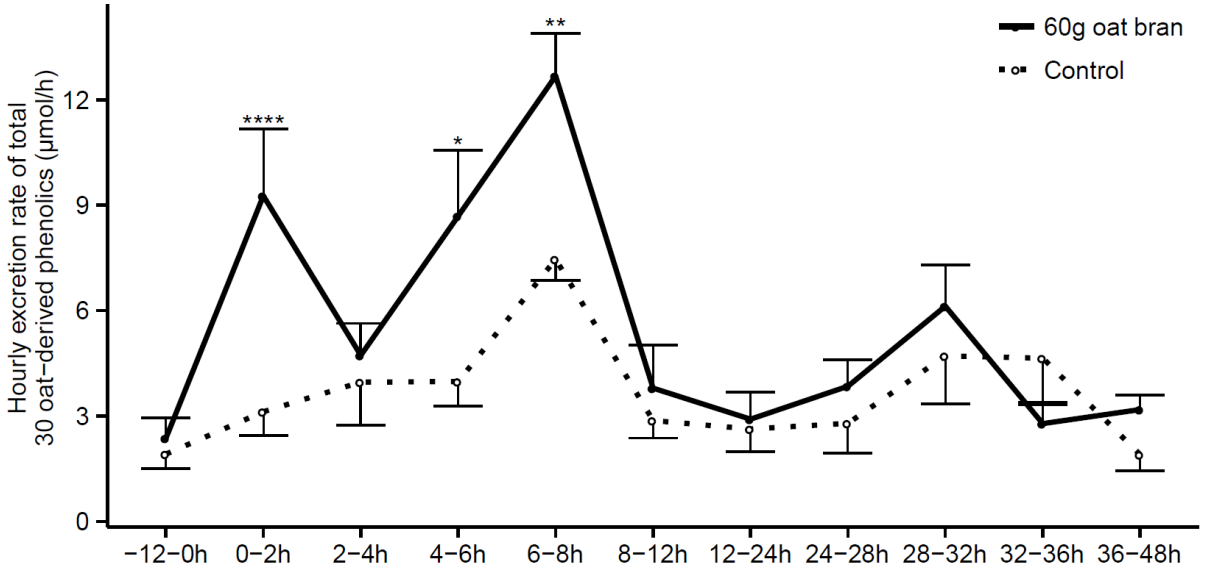

Figure 2: Urinary excretion rate of total 30 oat bran-derived phenolic compounds after intake of 60g oat bran or a control meal in healthy men (µmol/h). Data are reported as mean ± SEM and were analyzed by two-factor repeated measurement linear mixed model with time and intervention as the two factors [significant effect of intervention (P = 0.005), time ($P = 3*10^{-13}$), and time and intervention interaction ($P = 5*10^{-6}$)]. *Post-hoc* analysis with Tukey-Kramer adjustment was performed and P-values are indicated as follow: * P < 0.05; ** P <0.01; **** P<0.0001.

Hourly urinary excretion of oat phenolic compounds

At baseline, the mean hourly excretion of the total excretion of 30 phenolic compounds prior to oat bran and control interventions was 2.4±0.6 µmol/h and 1.9±0.4 µmol/h, respectively (Figure 2). Following intervention, the total hourly phenolic excretion significantly increased at 0-2h (oat: 9.3±1.9 µmol/h, control: 3.1±0.7 µmol/h; post-hoc P-value <0.0001), returned to a similar level as control at 2-4h (oat: 4.7±0.9 µmol/h, control: 4.0±1.2µmol/h; post-hoc P-value >0.05) and peaked again between the 4-6 h period (oat: 8.7±1.9µmol/h, control: 4.0±0.7µmol/h; post-hoc P-value < 0.05) and 6-8h (oat: 13.7±1.2µmol/h, control: 7.5±0.6µmol/h; post-hoc P-value < 0.01) before returning to a similar level to the control intervention from 8 to 48h (post-hoc P-value >0.05; Figure 2). Figure 3 shows the oat bran induced increase in excretion of the individual 30 oat bran-derived phenolics proportional to the total excretion at 0-2h, 4-6h and 6-8h, relative to control. Vanillic acid, 3-hydroxyhippuric acid, 4-hydroxyhippuric acid, benzoic acid-*O*-sulfate and ferulic acid- *O*-sulfate were the predominant oat bran-derived phenolics excreted, accounting collectively for more than two thirds of the total excretion (i.e. 20.3%, 16.3%, 16.1%, 9.8% and 7.3%, respectively). (Iso)ferulic acid-*O*-sulfate was predominant

during the early 0-2h peak, vanillic acid was almost exclusively excreted during the late 4-8h period, whilst excretion of hydroxyhippuric acids and benzoic acid-*O*-sulfate was biphasic, with early and late peaks (Figure 3). Ferulic acid, *p*-coumaric acid and avenanthramides 2p (i.e. the most abundant phenolic compounds in oats) accounted only for small percentages of the total excreted phenolics (i.e. 0.21%, 0.04% and 0.02%, respectively; Figure 3) and avenanthramide 2f and 2c were not detected, suggesting that these dietary forms are subject to extensive metabolism.

The mean absolute urinary excretion of the 30 oat bran-derived phenolics from 0-8h was 70.7±8.1µmol after consumption of oat bran and 37.1±4.4µmol after the control intervention (Table 2). Hence, on average 22.9±5.0% of the ingested dose (i.e. 147µmol phenolics in 60g oat bran; Table 1) was recovered in the 0-8h urine and there was large inter-individual variability in absorption that ranged from 3.7% to 41.2% (Table 2). The mean percentage recovery of metabolites was lowest for the avenanthramide 2p (0.3%), intermediate for hydroxycinnamic acids (6.8-10.7%) and highest for hydroxybenzoic acids (14.5-159.2%; Table 2).

Table 2: 0-8h absolute urinary excretion (µmol) of oat bran-derived phenolics and recovery of ingested dose (%)

	Mean±SEM urinary excretion 0-8h (µmol)			Recovery of ingested dose (%)		
Excreted phenolics	Control	Oat bran	Difference	Mean±SEM	Lowest/highest absorber	Oat parent compounds (Ingested dose)
Avenanthramide 2p	0.006±0.001	0.012±0.003	0.006±0.003	0.3 ± 0.1%	0% /0.7%	Avenanthramide 2p (2.2µmol)
Hydroxycinnamic acids						
Ferulic acid metabolites[a)]	4.1±0.4	10±0.8	5.9±0.7	6.8 ± 0.8%	4.9% /9.3%	Ferulic acid (86.5µmol)
Caffeic acid-O-sulfate	0.4±0.2	1.2±0.4	0.8±0.5	10.7 ± 7%	-15.6% /34.9%	Caffeic acid (7.4µmol)
Sinapic acid-O-sulfate	0.5±0.1	1.5±0.2	1±0.2	10.6 ± 1.6%	4.9% /15%	Sinapic acid (9.3µmol)
Hydroxybenzoic acids						
(Iso)vanillic acid	6.1±2.5	13.5±3.1	7.3±2.5	159.2 ± 53.8%	-80.5% /308.3%	Vanillic acid (4.6µmol)
Benzoic acid-O-sulfate/glucuronide	7.1±2	10.3±2.7	3.2±1	85.2 ± 26%	8.1% /176.1%	4-hydroxybenzoic acid (3.7µmol)
Syringic acid-O-sulfate	0.4±0.1	0.9±0.2	0.5±0.1	14.5 ± 4.1%	1.5% /30.5%	Syringic acid (3.6µmol)
Total 30 phenolics	37.1±4.4	70.7±8.1	33.7±7.3	22.9 ± 5%	3.7% /41.2%	Total 12 phenolics (147.1µmol)

[a)] Sum of ferulic, isoferulic, dihydroferulic and dihydroisoferulic acids: aglycones, glucuronides or sulfates

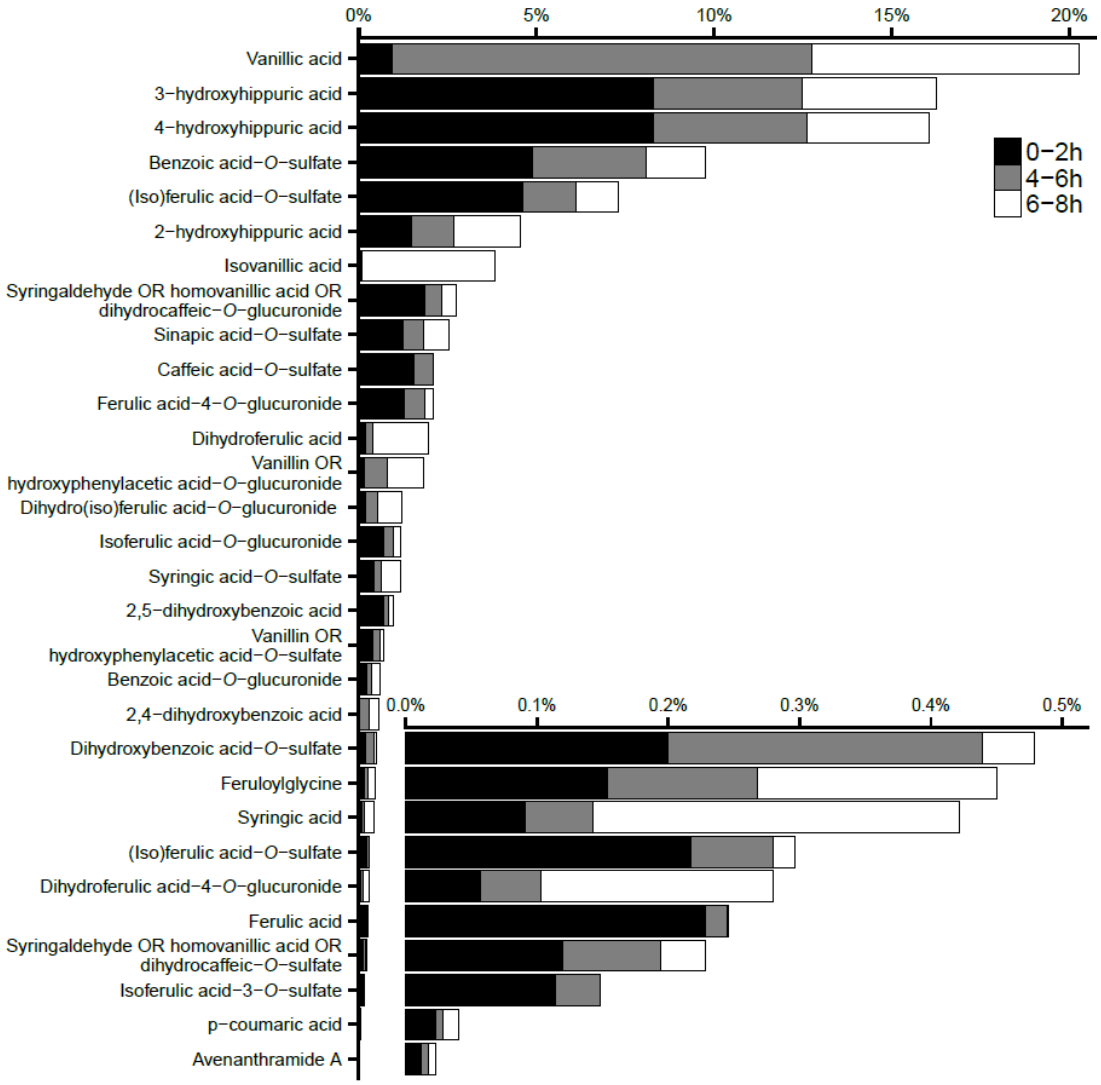

Figure 3: Proportions of individual oat bran-derived phenolics relative to total excretion levels at 0-2h, 4-6h and 6-8h. Excretion level differences between the oat and control interventions were used to calculate percentages.

Discussion

The present randomized controlled trial is, to our knowledge, the first to have examined the excretion of phenolic metabolites following intake of dietary amounts of oat bran as a whole food over a 48h timeframe and complements previous studies that examined the metabolism and bioavailability of phenolics following intake of an oat avenanthramide extract at a high-dose (123) or following intake of wheat (150). Oat bran intake resulted in elevated urinary excretion of 30 phenolic compounds at 0-2h and 4-8h, with sulfate or glycine conjugated benzoic acids being the major metabolites together with ferulic acid-*O*-sulfate, and the total amounts of phenolic compounds recovered in urine amounting to 22.9±5.0% of the ingested phenolic dose. These results suggest that a high proportion of oat phenolics are bioavailable as a wide range of metabolites with absorption occurring both in the small intestine and then in the large intestine within eight hours of consumption.

Analysis of the utilized oat bran detected nine phenolic acids amounting to 434 μg/g and three avenanthramides amounting to 41 μg/g (Table 1). These levels are comparable to four previous studies that analyzed wholegrain oats or oat bran, identifying up to 10 phenolic acids at levels ranging from 273 to 874 μg/g and three avenanthramides at levels ranging from 13 to 116 μg/g (296, 301, 396, 403). The relatively wide range in the reported contents and compositions of phenolics can be explained by the different oat products analyzed (i.e. commercial or non-commercial varieties, bran or wholegrain, and hulled or de-hulled), and by the different extraction and analytical methods used for their analysis. Although other cereals may be richer sources of phenolic compounds (for example, Mattila et al. found 6.8-fold and 6.3-fold more total phenolic compounds in wheat and rye bran, respectively, than in oat bran (301)), oats are particularly interesting because of the relatively high proportions of phenolics in free and conjugated forms (24% in our oat bran and from 34 to 62% in non-commercial hulled wholegrain oats (396), whereas wheat has less than 18% (402) and wheat bran cereals only 8% (151)) and thus phenolic components from oats may have a higher bio-accessibility in comparison to other cereals.

We observed high background excretion of all 30 oat bran-derived phenolic compounds at baseline and in the control arm (Supporting Information Table 3; Figure 2), even though volunteers followed a 48h low-phenolic diet. A high background phenolic excretion has previously been described in the control groups of other studies of (poly)phenol bioavailability after following 2d (404) or 7d (405) low phenolic dietary restrictions. These phenolic compounds may originate from the relatively low amounts of phenolics consumed in white bread and pasta during the dietary restrictions (402) and from the metabolism of other dietary components such as aromatic amino acids (406). Despite this high background excretion, our data indicate that intake of oat bran leads to urinary excretion of 30 phenolic acid compounds which peak early at 0-2h, and again later between 4-8h (Figure 2). The early appearance of metabolites suggests that the absorption of free phenolics from the oat bran occurs in

the upper gastrointestinal tract with esterified conjugates being hydrolyzed by esterase activity in the intestinal mucosa (407), followed by the transfer of free phenolics across the intestinal epithelium through passive diffusion or via transporters (408). Although we hypothesized that phenolics in the bound fraction would appear late in the urine, due to a requirement for gut microbiota to cleave covalent linkages between phenolic and fibre, the second peak of excretion was complete by 8 h, suggesting that the release and absorption occurred more rapidly (Figure 2). This agrees with previous studies, showing that bound phenolics may reach the colon and undergo microbial fermentation within 4 hours of oat bran intake. Indeed, in fasted volunteers (which was true in our study) a mean mouth-to-cecum transit time can be as rapid as 2.3h (409) and *in vitro* fermentation of wheat bran with human microbiota shows that digestion by microbial esterase and xylanase starts within 2h resulting in most fiber-bound phenolics being released within 6h (410).

The urinary recovery of ingested phenolics was on average 22.9±5.0% in the first 8 h after the oat bran intake, relative to the control (Table 2). Previous non-controlled studies reported lower mean recoveries in 24h urine of 4 ± 1%, 8 ± 2% and 3 ± 1% following ingestion of phenolics in wholegrain wheat bread (150), aleurone-enriched wheat bread (150) or wheat bran cereals (151), respectively, but a higher mean recovery of 29 ± 4% following ingestion of phenolics in instant coffee (400). This wide variation in phenolic bioavailability may partly be explained by differences in the bioaccessibility of phenolics within the food matrix. While phenolic acids in coffee are conjugated to quinic acids and are relatively water soluble (400), the oat bran used here had 24% soluble phenolic acids (Table A1) and wheat bran cereals only 9% (151). However, such direct comparisons of recoveries between studies may be of limited value due to the non-controlled design of previous studies (150, 151, 400), and differences in the methods used to analyze phenolic acids in the urine and food samples including the number of metabolites targeted with the LC-MS/MS methods.

Free forms of vanillic and isovanillic acids were the highest and 7th most highly excreted phenolics, respectively being mainly excreted in the late peak between 4-8h after oat bran intake (Figure 3) and at levels totaling more than the total amount of vanillic acid that was ingested (mean ± SEM recovery: 159.2 ± 53.8%; Table 2). These data suggest that whilst only a limited amount of vanillic acid is absorbed directly in the small intestine, a larger proportion is absorbed following release by fermentation by the colonic microbiota with previous studies suggesting that it originated partly from the metabolism of avenanthramides and hydroxycinnamic acids (404, 411-413). Vanillic acid may be formed by β-oxidation of ferulic acid in the liver, and isovanillic acid from methylation of caffeic acid to isoferulic acid followed by liver β-oxidation (404, 411-413). 4-, 3- and 2-hydroxyhippuric acids were also highly excreted, with their excretion following a biphasic pattern (early 0-2h and late 4-8h) after oat bran intake (Figure 3). While hydroxyhippuric acids are common flavonoid metabolites detected, for example, following intake of orange juice (414) and cocoa (415), results from this oat trial and a

recent wheat trial (150) suggest that hydroxyhippuric acids are also important metabolites derived from wholegrain.

A study feeding 75-150mg avenanthramides in an oat extract to humans detected nanomolar concentrations of circulating avenanthramides (123), while the present study detected only traces of the ingested 2.5mg avenanthramides in urine. This may indicate that avenanthramides are bioavailable when ingested at a relatively high dose and that avenanthramides are mostly metabolized particularly to hydroxycinnamic acids during their passage through the GI tract and into the circulation (Table 2). Hydroxycinnamic acids, in turn, undergo reduction, methylation, sulfation or glucuronidation, and are also metabolized to smaller hydroxybenzoic acids (Table 2). Chlorogenic acid and caffeic acid aglycones in coffee have a similar metabolic fate to avenanthramides and hydroxycinnamic acids in cereals. A study in ileostomy patients showed that although 33% of chlorogenic acid and 95% of caffeic acid were absorbed in the small intestine, only traces of chlorogenic acid and 11% of caffeic acid of the ingested dose were excreted in the urine (416). Avenanthramides were also metabolize to hydroxyanthralinic acid and dihydroavenanthramides as reported in a study feeding in mice with a high dose of 200mg/kg avenanthramide 2c (313); however, the present study did determine these two compounds because of poor ionization of the former and unavailability of sMRM parameters for the latter.

In conclusion, oats are a popular health food and to our knowledge the present study is the first to study the metabolic fate of oat avenanthramides and phenolic acids when ingested at a dietary dose and as a whole food. Our data suggest that oat bran phenolics are more bioavailable than previous studies reported (150, 151, 400) with 22.9±5.0% of the ingested dose being excreted in urine during 8h following intake in the form of 30 different phenolics or their metabolites. The data showed that benzoic acid derivatives, and in particular (iso)vanillic acid, and three isoforms of hydroxyhippuric acids, accounted for a high proportion of the excreted compounds, together with ferulic acid-*O*-sulfate. While the present study is an important contribution to the existing literature, future work is required to verify tentatively identified phenolic metabolites against analytical standards, to establish the detailed pharmacokinetics and circulating concentrations of these oat bran-derived phenolic compounds, and to determine their biological activities and contributions to the health benefits of a diet rich in oats.

Supplementary Material

Supporting Information Table 1 – Common and IUPAC names of phenolic compounds

Common Name	IUPAC Name
List of available analytical standards	
2,4-dihydroxy benzoic acid	2,4-dihydroxybenzoic acid
2,5-dihydroxy benzoic acid	2,5-dihydroxy benzoic acid
2-hydroxyhippuric acid	2-[(2-hydroxybenzoyl)amino]acetic acid
3-hydroxyhippuric acid	2-[(3-hydroxybenzoyl)amino]acetic acid
4-hydroxybenzaldehyde	4-hydroxybenzaldehyde
4-hydroxybenzoic acid	4-hydroxybenzoic acid
4-hydroxyhippuric acid	2-[(4-hydroxybenzoyl)amino]acetic acid
4-hydroxyphenylacetic acid	2-(4-hydroxyphenyl)acetic acid
5-hydroxyanthranilic acid	2-amino-5-hydroxybenzoic acid
Avenanthramide 2c	2-[[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]amino]-5-hydroxybenzoic acid
Avenanthramide 2f	5-hydroxy-2-[[(E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]amino]benzoic acid
Avenanthramide 2p	5-hydroxy-2-[[(E)-3-(4-hydroxyphenyl)prop-2-enoyl]amino]benzoic acid
Caffeic acid	(E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid
Dihydrocaffeic acid	3-(3,4-dihydroxyphenyl)propanoic acid
Dihydroferulic acid	3-(4-hydroxy-3-methoxyphenyl)propanoic acid
Dihydroferulic acid-4-*O*-glucuronide	(2S,3S,4S,5R,6S)-6-[4-(2-carboxyethyl)-2-methoxyphenoxy]-3,4,5-trihydroxyoxane-2-carboxylic acid
Dihydroxybenzoic acid-3-*O*-glucuronide	(2S,3S,4S,5R,6S)-6-(5-carboxy-2-hydroxyphenoxy)-3,4,5-trihydroxyoxane-2-carboxylic acid
Ferulic acid	(E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid
Ferulic acid-4-*O*-glucuronide	(2S,3S,4S,5R,6S)-6-[4-[(E)-2-carboxyethenyl]-2-methoxyphenoxy]-3,4,5-trihydroxyoxane-2-carboxylic acid
Gallic acid	3,4,5-trihydroxybenzoic acid
Hippuric acid	2-benzamidoacetic acid
Homovanillic acid	2-(4-hydroxy-3-methoxyphenyl)acetic acid
Isoferulic acid	(E)-3-(3-hydroxy-4-methoxyphenyl)prop-2-enoic acid
Isoferulic acid-3-*O*-sulfate	(E)-3-[3-sulfooxy-4-(methoxy)phenyl]prop-2-enoic acid
Isovanillic acid	3-hydroxy-4-methoxybenzoic acid
o-coumaric acid	(E)-3-(2-hydroxyphenyl)prop-2-enoic acid
p-coumaric acid	(E)-3-(4-hydroxyphenyl)prop-2-enoic acid
Protocatechuic acid	3,4-dihydroxybenzoic acid

Salicylic acid	2-hydroxybenzoic acid
Sinapic acid	(E)-3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid
Syringaldehyde	4-hydroxy-3,5-dimethoxybenzaldehyde
Syringic acid	4-hydroxy-3,5-dimethoxybenzoic acid
Vanillic acid	4-hydroxy-3-methoxybenzoic acid
Vanillin	4-hydroxy-3-methoxybenzaldehyde
Tentatively identified oat-derived phenolics	
3,4-dihydroxyhydrocinamic acid-*O*-glucuronide	(2S,3S,4R,5R,6S)-3-(5-(2-carboxyethyl)-2-hydroxyphenoxy)-4,5,6-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
4-hydroxyphenylacetic acid-*O*-glucuronide	(2S,4R,5R,6S)-3-(4-(carboxymethyl)phenoxy)-4,5,6-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Benzoic acid-*O*-glucuronide	(2S,3S,4R,5R,6S)-3-(4-carboxyphenoxy)-4,5,6-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Benzoic acid-O-sulfate	4-carboxyphenyl sulfate
Caffeic acid-O-sulfate	(E)-4-(2-carboxyvinyl)-2-hydroxyphenyl sulfate
Dihydroferulic acid-O-glucuronide	(2S,3S,4R,5R,6S)-3-(4-(2-carboxyethyl)-2-methoxyphenoxy)-4,5,6-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Dihydroxybenzoic acid-O-sulfate	4-carboxy-2-hydroxyphenyl sulfate
Feruloylglycine	2-[[(E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]amino]acetic acid
Homovanillic acid-O-glucuronide	(2S,3S,4S,5R)-6-(4-(carboxymethyl)-2-methoxyphenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Hydroxyphenylacetic acid-O-sulfate	4-(carboxymethyl)phenyl sulfate
(Iso)ferulic acid-O-glucuronide	(2S,3S,4R,5R,6S)-3-(4-((E)-2-carboxyvinyl)-2-methoxyphenoxy)-4,5,6-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
(Iso)ferulic acid-O-sulfate	4-(2-carboxyethyl)-2-methoxyphenyl sulfate
Sinapic acid-O-sulfate	(E)-4-(2-carboxyvinyl)-2,6-dimethoxyphenyl sulfate
Syringaldehyde-O-glucuronide	(2S,3S,4R,5R,6S)-3-(4-formyl-2,6-dimethoxyphenoxy)-4,5,6-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Syringaldehyde-O-sulfate	4-formyl-2-hydroxy-6-methoxyphenyl sulfate
Syringic acid-O-sulfate	4-carboxy-2,6-dimethoxyphenyl sulfate
Vanillin-O-glucuronide	(2S,3S,4R,5R,6S)-3-(4-formyl-2-methoxyphenoxy)-4,5,6-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Vanillin-O-sulfate	4-formyl-2-methoxyphenyl sulfate
Non oat-derived phenolics	
3,4-dihydrocaffeic acid	3-(3,4-dihydroxyphenyl)propanoic acid
4-hydroxybenzaldehyde	4-hydroxybenzaldehyde
4-hydroxyphenylacetic acid	2-(4-hydroxyphenyl)acetic acid
3-hydroxyphenylacetic acid	2-(3-hydroxyphenyl)acetic acid
Dihydroisoferulic acid	3-(3-hydroxy-4-methoxyphenyl)propanoic acid
Isoferulic acid	(E)-3-(3-hydroxy-4-methoxyphenyl)prop-2-enoic acid
o-coumaric acid	(E)-3-(2-hydroxyphenyl)prop-2-enoic acid
Salicylic acid	2-hydroxybenzoic acid
Syringaldehyde	4-hydroxy-3,5-dimethoxybenzaldehyde
Vanillin	4-hydroxy-3-methoxybenzaldehyde
4-hydroxybenzoic acid	4-hydroxybenzoic acid
Caffeic acid	(E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid

Gallic acid	3,4,5-trihydroxybenzoic acid
Hippuric acid	2-benzamidoacetic acid
Homovanillic acid	2-(4-hydroxy-3-methoxyphenyl)acetic acid
Protocatechuic acid OR acid	3,4-dihydroxybenzoic acid
3,5-dihydroxybenzoic	3,5-dihydroxybenzoic
Salicylic acid-O-sulfate	2-sulfooxybenzoic acid
Dihydroxybenzoic acid-O-sulfate	3-carboxy-2-hydroxyphenyl sulfate
Coumaric acid-O-sulfate	(E)-3-(4-sulfonatooxyphenyl)prop-2-enoate
(iso)vanillic acid-O-sulfate	4-carboxy-2-methoxyphenyl sulfate
Hippuric acid-O-sulfate	(2-(sulfooxy)benzoyl)glycine
Homovanillic acid-O-sulfate	2-(3-methoxy-4-(sulfooxy)phenyl)acetic acid
Dihydroxyhydroycinamic acid-O-sulfate	3-(2-carboxy-2,2-dihydroxyethyl)phenyl sulfate
Hydroxybenzaledhyde-O-glucuronide	(2S,3S,4S,5R)-6-(2-formyl-6-hydroxyphenyl)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-carboxylic acid
Benzoic acid-O-glucuronide	(2S,3S,4S,5R)-6-(2-carboxyphenyl)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-carboxylic acid
Vanillin-O-glucuronide	(2S,3S,4S,5R)-6-(4-formyl-2-methoxyphenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Hydroxyphenylacetic acid-O-glucuronide	(2S,3S,4S,5R)-6-(carboxy(phenyl)methoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Coumaric acid-O-glucuronide	(2S,3S,4S,5R)-6-(4-((E)-2-carboxyvinyl)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
(iso)vanillic acid-O-glucuronide	(2S,3S,4S,5R)-6-(4-carboxy-2-methoxyphenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Hippuric acid-O-glucuronide	(2S,3S,4S,5R)-6-(2-((carboxymethyl)carbamoyl)phenyl)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-carboxylic acid
Caffeic acid-O-glucuronide	(2S,3S,4S,5R)-6-(5-((E)-2-carboxyvinyl)-2-hydroxyphenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Homovanillic acid-O-glucuronide	(2S,3S,4S,5R)-6-(4-(carboxymethyl)-2-methoxyphenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Syringaldehyde-O-glucuronide	(2S,3S,4S,5R)-6-(4-formyl-2,6-dimethoxyphenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Dihydroxycaffeic acid-O-glucuronide	(2S,3S,4S,5R)-6-(4-(2-carboxy-2,2-dihydroxyethyl)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Dihydroferulic acid-O-glucuronide	(2S,3S,4S,5R)-6-(4-(2-carboxyethyl)-2-methoxyphenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid
Syringic acid-O-glucuronide	(2S,3S,4S,5R)-6-(4-carboxy-2,6-dimethoxyphenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid

Supporting Information Table 2 – sMRM transitions, parameters and detection limits of 30 identified oat bran-derived phenolics

Metabolite	RT	MW	sMRM ion transitions (m/z)	Collision energy (V)	LOD (nM)	LOQ (nM)	R^2
Aglycones							
2,5-dihydroxybenzoic acid	4.8	154.1	153 / 109	-11	4.7	15.8	0.999
2,4-dihydroxybenzoic acid	5.5	154.1	153 / 109	-11	7.3	24.3	0.999
Vanillic acid	5.6	168.2	169 / 93	12	9.8	32.7	0.994
Isovanillic acid	6.0	168.2	169 / 93	12	7.3	24.4	0.999
Syringic acid	6.5	198.2	197 / 182	-13	14.9	49.6	0.999
Dihydroferulic acid	7.1	196.2	195 / 136	-15	21.5	71.8	1.000
p-coumaric acid	7.3	164.2	163 / 119	-13	4.2	9.5	0.997
Ferulic acid	7.7	194.2	193 / 178	-10	8.2	27.4	1.000
Avenanthramide 2p	9.9	299.3	298 / 254	-17	0.8	2.7	0.994
Glycines							
4-hydroxyhippuric acid	4.4	195.2	194 / 100	-11	4.3	14.2	0.997
3-hydroxyhippuric acid	4.7	195.2	194 / 100	-11	24.5	81.7	0.999
2-hydroxyhippuric acid	7.0	195.2	194 / 100	-11	2.9	9.7	0.998
Feruloylglycine	6.4	251.2	250 / 100*, 206, 191, 177, 149	-11	2.2	7.2	no standard
Glucuronides							
Syringaldehyde OR Homovanillic acid OR 3,4-dihydroxyhydrocinamic acid-*O*-glucuronide[a,b)]	4.2	358.2	357 / 113* , 59 , 137 , 175 , 181	-17, -23, -23, -11, -17	20.7	68.9	no standard
Benzoic acid-*O*-glucuronide[a)]	5.1	314	313 / 113* , 175 , 137 , 93	-17, -17, -17, -23	15.7	52.2	no standard
Ferulic Acid-4-*O*-Glucuronide	5.3	370.3	369 / 193* , 178 , 113 , 175	-19, -23, -19, -10	21.4	71.3	0.997
Vanillin OR 4-hydroxyphenylacetic acid-*O*-glucuronide[a,b)]	5.3	328.1	327 / 113* , 107 , 175 , 151	-17, -23, -11, -11	35.0	116.7	no standard
Dihydroferulic acid-4-*O*-glucuronide	5.8	372.3	371 / 113* , 195 , 85 , 175	-17, -20, -23, -17	11.7	39.1	0.990
Isoferulic acid-*O*-glucuronide[a)]	6.1	370	369 / 193* , 178 , 113 , 175	-19, -23, -19, -10	11.2	37.5	no standard
Dihydroferulic acid-*O*-glucuronide[a)]	6.2	372.3	371 / 113* , 195 , 85 , 175	-17, -20, -23, -17	8.0	26.5	no standard
Sulfates							
Dihydroxybenzoic acid-*O*-sulfate[a)]	3.0	234	233 / 109* , 189, 153, 97	-23, -11, -23, -23	3.6	12.1	no standard
Benzoic acid-*O*-sulfate[a)]	3.3	218	217 / 137*, 173	-11, -11	5.7	19.3	no standard
Syringaldehyde-*O*-sulfate[a)]	4.1	262.2	261 / 166* , 123 , 181	-23, -23, -11	3.1	10.2	no standard
Syringic acid-*O*-sulfate[a)]	4.1	278.2	277 / 197* , 182 , 153 , 123	-11, -23, -23, -23	2.6	8.8	no standard
Caffeic acid-*O*-sulfate[a)]	5.4	260.2	259 / 179* , 135 , 107	-17, -23, -23	7.7	25.8	no standard
Sinapic acid-*O*-sulfate[a)]	5.6	304.2	303 / 223* , 208 , 164 , 120	-11, -23, -23, -23	1.6	5.3	no standard
(Iso)ferulic acid-*O*-sulfate[a)]	5.7	274.2	273 / 178* , 192 , 134	-20, -16, -25	1.6	5.3	no standard
(Iso)ferulic acid-*O*-sulfate[a)]	5.9	274.2	273 / 178* , 192 , 134	-20, -16, -25	1.5	5.0	no standard
Isoferulic acid 3-*O*-sulfate	6.1	274.2	273 / 178* , 192 , 134	-20, -16, -25	1.4	4.7	1.000
Vanillin or hydroxyphenylacetic acid-*O*-sulfate[a)]	6.4	232.1	231.1 / 151*, 136, 92	-17, -23, -23	2.7	8.9	no standard

*sMRM transition used for quantification; [a)] site of conjugation could not be ascertained as identification was based on known transitions where pure standards for isomers were not available or separation of isomers was chromatographically not possible; [b)]tentatively identified compound which could have different isomeric configuration. Abbreviations: LOD, limit of detection (Signal/Noise = 3); LOQ, limit of quantification (Signal/Noise = 10); MW, molecular weight; RT, Retention time; sMRM, scheduled multiple reaction monitoring; R^2, linear regression coefficient of standard curve; no standard, no analytical standard was available to make standard curve

Supporting Information Table 3 – Urinary excretion rate per hour of oat bran-derived phenolic compounds after intake of 60g oat bran or a control meal in six healthy men (nmol/h)

Metabolite	Meal	-12-0h	0-2h	2-4h	4-6h	6-8h	8-12h	12-24h	24-28h	28-32h	32-36h	36-48h	Model P value		
													Inter-vention	Time	Inter-action
								µmol/h							
Avenanthramide A	Oats	1 ± 0	3 ± 0****	1 ± 0	2 ± 0*	2 ± 0	1 ± 0	1 ± 0	2 ± 0	1 ± 0	1 ± 0	1 ± 0	***	***	***
	CON	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	2 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0			
Ferulic acid	Oats	9 ± 4	61 ± 15*	29 ± 6	21 ± 3	41 ± 9	15 ± 5	15 ± 8	15 ± 3	24 ± 8	12 ± 4	13 ± 3	0.3	****	*
	CON	7 ± 1	21 ± 4	13 ± 4	18 ± 6	48 ± 23	25 ± 13	24 ± 13	14 ± 3	26 ± 5	23 ± 9	8 ± 3			
p-coumaric acid	Oats	1 ± 0	7 ± 1**	3 ± 0	3 ± 1	5 ± 0	2 ± 1	2 ± 0	2 ± 1	2 ± 0	2 ± 1	1 ± 0	0.1	***	*
	CON	2 ± 1	3 ± 1	2 ± 1	2 ± 0	3 ± 0	3 ± 2	3 ± 1	1 ± 0	2 ± 0	2 ± 1	1 ± 0			
Dihydroferulic acid	Oats	34 ± 16	90 ± 42	34 ± 12	60 ± 17	378 ± 135****	109 ± 68	35 ± 17	23 ± 5	78 ± 22	60 ± 19	23 ± 6	0.1	****	***
	CON	14 ± 5	56 ± 28	26 ± 8	26 ± 15	102 ± 38	38 ± 24	32 ± 10	24 ± 5	101 ± 66	84 ± 55	18 ± 5			
Isovanillic acid	Oats	6 ± 5	24 ± 22	44 ± 5	69 ± 18	744 ± 470***	54 ± 51	22 ± 20	16 ± 3	188 ± 136	54 ± 44	31 ± 15	0.1	*	*
	CON	7 ± 6	18 ± 17	30 ± NA	62 ± 14	87 ± 39	4 ± 1	1 ± 0	ND	59 ± 17	4 ± 1	1 ± 0			
Syringic acid	Oats	6 ± 2	29 ± 5	12 ± 3	18 ± 5	65 ± 20****	23 ± 10	6 ± 2	12 ± 2	14 ± 3	8 ± 4	6 ± 1	*	****	***
	CON	2 ± 1	13 ± 3	12 ± 3	9 ± 1	16 ± 3	9 ± 2	8 ± 1	19 ± 7	14 ± 3	13 ± 4	4 ± 1			
2,4-dihydroxybenzoic acid	Oats	4 ± 1	15 ± 1	10 ± 3	54 ± 26*	62 ± 18***	13 ± 5	23 ± 15	12 ± 2	43 ± 22	10 ± 3	8 ± 2	**	**	***
	CON	4 ± 1	11 ± 3	16 ± 6	6 ± 2	16 ± 5	6 ± 2	5 ± 2	18 ± 8	6 ± 2	6 ± 2	4 ± 2			
2,5-dihydroxybenzoic acid	Oats	51 ± 17	209 ± 58**	78 ± 9	87 ± 15	97 ± 13	41 ± 11	27 ± 9	32 ± 10	28 ± 6	17 ± 6	27 ± 11	0.3	****	*
	CON	47 ± 9	88 ± 23	70 ± 24	63 ± 21	73 ± 15	34 ± 11	29 ± 16	40 ± 24	34 ± 19	43 ± 31	20 ± 8			
Vanillic acid	Oats	29 ± 14	175 ± 36	80 ± 8	3352 ± 743**	2918 ± 624*	201 ± 98	58 ± 22	39 ± 12	1600 ± 600	168 ± 71	56 ± 21	*	****	*
	CON	13 ± 8	13 ± 7	41 ± 14	1281 ± 624	1594 ± 702	45 ± 33	15 ± 11	28 ± 5	1162 ± 513	128 ± 37	30 ± 20			
2-hydroxyhippuric acid	Oats	111 ± 35	371 ± 173*	133 ± 35	294 ± 112	525 ± 82***	228 ± 75	137 ± 28	198 ± 107	133 ± 37	81 ± 21	117 ± 27	**	****	**
	CON	101 ± 21	113 ± 29	121 ± 32	84 ± 17	195 ± 20	101 ± 21	70 ± 24	54 ± 20	99 ± 41	158 ± 55	58 ± 20			
3-hydroxyhippuric acid	Oats	443 ± 148	2068 ± 536**	958 ± 278	1142 ± 409	1643 ± 302	414 ± 124	452 ± 98	599 ± 209	650 ± 329	288 ± 75	557 ± 98	*	****	**
	CON	395 ± 89	617 ± 203	729 ± 226	405 ± 140	981 ± 271	364 ± 97	541 ± 298	344 ± 223	297 ± 111	820 ± 325	355 ± 126			
4-hydroxyhippuric acid	Oats	553 ± 133	2262 ± 551**	1289 ± 289	1566 ± 457	2479 ± 79	939 ± 348	748 ± 187	1108 ± 274	1302 ± 298	885 ± 228	899 ± 105	0.1	****	**
	CON	430 ± 71	809 ± 232	1133 ± 342	807 ± 195	1875 ± 246	820 ± 105	628 ± 221	650 ± 247	1020 ± 334	1314 ± 307	528 ± 167			
Feruloylglycine	Oats	11 ± 5	39 ± 12	11 ± 2	28 ± 8	56 ± 18**	22 ± 10	13 ± 3	8 ± 2	15 ± 5	11 ± 4	12 ± 3	0.1	****	***
	CON	9 ± 3	12 ± 3	9 ± 3	8 ± 1	24 ± 6	14 ± 3	14 ± 4	9 ± 3	19 ± 5	28 ± 10	8 ± 2			
Caffeic acid-sulfate	Oats	68 ± 31	298 ± 127***	90 ± 38	128 ± 41	103 ± 25	23 ± 10	18 ± 7	13 ± 4	34 ± 15	17 ± 5	12 ± 4	0.6	0.1	***
	CON	65 ± 27	28 ± 8	15 ± 5	30 ± 12	137 ± 98	110 ± 84	47 ± 19	18 ± 6	76 ± 51	103 ± 62	22 ± 9			
(Iso)ferulic acid-*O*-sulfate	Oats	78 ± 25	879 ± 142****	560 ± 104	474 ± 57	661 ± 119	245 ± 82	101 ± 36	170 ± 39	359 ± 76	219 ± 71	118 ± 29	****	****	****
	CON	48 ± 14	71 ± 20	282 ± 63	210 ± 51	454 ± 87	135 ± 31	79 ± 22	121 ± 54	235 ± 74	131 ± 58	70 ± 25			
(Iso)ferulic acid-*O*-sulfate	Oats	6 ± 1	43 ± 7****	29 ± 8**	20 ± 4	28 ± 6	12 ± 5	8 ± 1	8 ± 1	16 ± 3	10 ± 3	7 ± 1	**	****	****
	CON	5 ± 2	5 ± 1	13 ± 3	9 ± 1	25 ± 4	9 ± 2	6 ± 1	7 ± 2	15 ± 4	14 ± 4	5 ± 1			
Isoferulic acid-3-*O*-sulfate	Oats	2 ± 0	23 ± 3****	16 ± 5	12 ± 1	7 ± 1	11 ± 6	3 ± 1	1 ± 0	4 ± 1	4 ± 2	3 ± 2	0.1	***	****
	CON	1 ± 0	3 ± 1	9 ± 4	6 ± 2	12 ± 7	4 ± 2	2 ± 1	6 ± 3	7 ± 4	6 ± 3	3 ± 1			
Syringic acid-*O*-sulfate	Oats	19 ± 8	99 ± 20***	54 ± 9	51 ± 10	165 ± 54****	76 ± 29	33 ± 11	41 ± 8	47 ± 9	32 ± 7	23 ± 4	**	****	****

	CON	15 ± 6	22 ± 9	58 ± 15	20 ± 4	68 ± 16	26 ± 9	22 ± 8	35 ± 12	51 ± 16	52 ± 16	13 ± 3			
Sinapic acid-*O*-sulfate	Oats	40 ± 15	249 ± 55****	108 ± 14	144 ± 21	252 ± 75**	66 ± 23	51 ± 17	55 ± 13	103 ± 20	49 ± 11	34 ± 6	**	****	****
	CON	28 ± 15	31 ± 13	59 ± 12	41 ± 8	130 ± 17	57 ± 25	45 ± 15	40 ± 11	101 ± 33	78 ± 27	32 ± 10			
Dihydroxybenzoic acid-*O*-sulfate	Oats	24 ± 6	53 ± 13*	27 ± 7	51 ± 12***	35 ± 7	16 ± 4	22 ± 6	16 ± 1	18 ± 4	18 ± 5	25 ± 4	0.2	0.1	****
	CON	16 ± 5	18 ± 2	14 ± 5	9 ± 2	28 ± 6	23 ± 8	26 ± 8	11 ± 2	18 ± 6	37 ± 18	17 ± 6			
Benzoic acid-*O*-sulfate	Oats	596 ± 209	1697 ± 494	720 ± 219	1230 ± 451	1283 ± 274	1062 ± 359	863 ± 351	1034 ± 274	1125 ± 368	580 ± 236	852 ± 270	0.5	*	*
	CON	506 ± 194	842 ± 251	935 ± 446	668 ± 213	988 ± 207	707 ± 162	819 ± 149	1073 ± 295	1146 ± 444	1066 ± 464	461 ± 143			
Vanillin or hydroxyphenylacetic acid-*O*-sulfate	Oats	13 ± 9	81 ± 50***	22 ± 12	40 ± 23	29 ± 15	12 ± 7	14 ± 6	20 ± 11	13 ± 6	9 ± 3	9 ± 3	0.3	*	*
	CON	4 ± 1	11 ± 7	6 ± 3	6 ± 3	9 ± 4	6 ± 3	7 ± 3	9 ± 5	7 ± 3	6 ± 2	7 ± 4			
Syringaldehyde OR homovanillic acid OR dihydroxyhydrocinamic acid sulfate	Oats	13 ± 3	38 ± 9*	21 ± 6	33 ± 9	46 ± 10	21 ± 8	15 ± 4	19 ± 2	32 ± 6	19 ± 4	14 ± 2	0.1	****	*
	CON	12 ± 3	17 ± 3	29 ± 8	20 ± 3	40 ± 11	15 ± 2	13 ± 2	18 ± 4	32 ± 9	27 ± 9	9 ± 1			
Ferulic Acid-4-*O*-glucuronide	Oats	49 ± 20	265 ± 65****	205 ± 36	170 ± 27	214 ± 33	62 ± 23	61 ± 22	57 ± 14	128 ± 30	80 ± 28	52 ± 11	****	****	****
	CON	45 ± 12	44 ± 10	85 ± 20	67 ± 12	171 ± 21	75 ± 19	58 ± 9	45 ± 16	123 ± 25	101 ± 40	38 ± 8			
Isoferulic acid-*O*-glucuronide	Oats	22 ± 6	142 ± 34****	94 ± 29	78 ± 14	104 ± 23	36 ± 32	23 ± 5	29 ± 7	58 ± 11	43 ± 15	25 ± 4	*	****	***
	CON	17 ± 4	21 ± 5	39 ± 9	29 ± 5	67 ± 8	33 ± 6	20 ± 3	20 ± 6	57 ± 7	41 ± 9	15 ± 4			
Dihydroferulic acid-4-*O*-glucuronide	Oats	3 ± 1	16 ± 5	11 ± 3	16 ± 5	49 ± 22****	27 ± 11	8 ± 4	8 ± 3	10 ± 2	10 ± 3	8 ± 3	**	***	*
	CON	3 ± 1	6 ± 3	6 ± 1	8 ± 3	18 ± 6	7 ± 4	5 ± 2	5 ± 1	12 ± 7	13 ± 8	7 ± 2			
Dihydro(iso)ferulic acid-*O*-glucuronide	Oats	31 ± 10	64 ± 16	21 ± 5	73 ± 20	189 ± 64****	69 ± 30	42 ± 12	29 ± 8	45 ± 10	53 ± 13	36 ± 9	*	****	**
	CON	27 ± 8	29 ± 6	14 ± 3	17 ± 5	68 ± 25	46 ± 15	35 ± 7	21 ± 4	39 ± 18	92 ± 44	27 ± 12			
Benzoic acid-*O*-glucuornide	Oats	23 ± 8	83 ± 26	28 ± 5	45 ± 15	78 ± 15**	36 ± 11	34 ± 7	37 ± 14	29 ± 6	19 ± 4	23 ± 5	***	****	*
	CON	27 ± 8	43 ± 10	31 ± 7	22 ± 4	36 ± 5	19 ± 3	23 ± 5	24 ± 9	25 ± 3	30 ± 12	19 ± 9			
Syringaldehyde OR Homovanillic acid OR 3,4-dihydroxyhydrocinamic acid-*O*-glucuronide	Oats	128 ± 30	528 ± 146**	205 ± 37	176 ± 53	273 ± 67	155 ± 31	167 ± 30	267 ± 60	142 ± 40	179 ± 47	203 ± 29	0.8	***	**
	CON	68 ± 27	201 ± 66	208 ± 61	95 ± 15	203 ± 40	141 ± 37	177 ± 34	179 ± 37	131 ± 32	211 ± 46	131 ± 32			
Vanillin OR 4-hydroxyphenylacetic acid-*O*-glucuronide	Oats	14 ± 7	39 ± 16	48 ± 18	134 ± 36****	212 ± 27****	60 ± 22	26 ± 8	19 ± 3	20 ± 6	16 ± 3	14 ± 3	***	****	****
	CON	10 ± 6	15 ± 7	22 ± 20	15 ± 5	36 ± 8	15 ± 2	17 ± 2	12 ± 2	19 ± 3	20 ± 4	11 ± 4			

Data are reported as mean ± SEM and were analysed by two-factor repeated measurement linear mixed model with time and treatment as the two factors. When the interaction effect was significant, post-hoc analyses with Tukey-Kramer adjustment were performed. If excretion was ND, the concentration was replaced with the limit of detection for statistical analysis. Model P values in the three far right columns and post-hoc P values next to the excretion rates are indicated as follow: * P < 0.05; ** P <0.01; *** P<0.001; **** P<0.0001. ND; not detected; CON control.

Supporting Information Table 4 – sMRM transitions, parameters and detection limits of non oat bran-derived phenolics

Metabolite	RT	MW	sMRM ion transitions (m/z)	Collision energy (V)	LOD (nM)	LOQ (nM)	R^2
3,4-dihydrocaffeic acid	6.4	182.17	181.17 / 137	-10	17.9	59.7	0.993
4-hydroxybenzaldehyde	5.9	122.12	121.12 / 92	-20	31.7	105.8	0.995
4-hydroxyphenylacetic acid	6.5	152.15	151.14 / 107	-10	56.3	187.8	0.997
3-hydroxyphenylacetic acid	6.3	152.15	151.14 / 107	-10	64.8	215.9	0.996
Dihydroisoferulic acid	7.5	196.2	195 / 136	-15	35.2	117.5	1.000
Isoferulic acid	8.0	195.2	194.2 / 136	-15	16.1	53.8	1.000
o-coumaric acid	8.4	164.2	163 / 119	-13	44.8	149.4	0.997
Salicylic acid	8.1	138.1	137.12 / 93	-23	41.0	136.6	0.999
Syringaldehyde	7.5	182.2	181.17 / 151	-18	0.3	1.0	0.993
Vanillin	6.9	152.1	151.15 / 136	-12	42.8	142.8	0.999
4-hydroxybenzoic acid	4.9	138.1	137.12 / 93	-13	64.8	216.1	0.998
Caffeic acid	6.0	180.2	179.16 / 135	-15	124.1	413.7	0.988
Gallic acid	1.7	170.1	169.12 / 125	-13	6.5	21.7	0.998
Hippuric acid	5.7	179.2	178.17 / 134	-11	211.6	705.5	0.995
Homovanillic acid	6.2	182.2	181.17 / 137	-10	44.4	148.0	0.999
Protocatechuic acid OR 3,5-dihydroxybenzoic acid[a)]	3.7	154.1	153.12 / 109	-13	64.1	213.7	0.998
Salicylic acid-*O*-sulfate [a,b)]	3.2	218.1	217 / 137* , 93 , 79	-11, -23, -17	75.5	251.6	no standard
Dihydroxybenzoic acid-*O*-sulfate [a,b)]	2.7	234.0	233 / 153* , 189 , 109	-23, -11, -23	76.8	255.9	no standard
Coumaric acid-*O*-sulfate [a,b)]	9.0	244.2	243 / 163* , 93 , 79	-17, -23, -23	38.0	126.6	no standard
(iso)vanillic acid-*O*-sulfate [a,b)]	4.2	248.0	247 / 167* , 152 , 123, 108	-23, -17, -17, -23	80.4	267.9	no standard
Hippuric acid-*O*-sulfate [a,b)]	3.4	259.2	258 / 178* , 79 , 134	-17, -23, -23	77.2	257.3	no standard
Hippuric acid-*O*-sulfate [a,b)]	3.1	259.2	258 / 178* , 79 , 134	-17, -23, -23	59.4	198.1	no standard
Hippuric acid-*O*-sulfate [a,b)]	3.8	259.2	258 / 178* , 79 , 134	-17, -23, -23	38.4	127.9	no standard
Homovanillic acid OR dihydrocaffeic acid-*O*-sulfate [a,b)]	4.6	262.2	261 / 181* , 121 , 79, 137	-17, -23, -23, -23	121.1	403.6	no standard
Homovanillic acid OR dihydrocaffeic-*O*-sulfate [a,b)]	4.9	262.2	261 / 181* , 121 , 79, 137	-17, -23, -23, -23	155.5	518.2	no standard
Hydroxybenzaledhyde-*O*-glucuronide [a,b)]	8.3	298.1	297 / 113* , 175 , 121	-11, -11, -23	50.8	169.2	no standard
Benzoic acid-*O*-glucuronide [a,b)]	2.7	314.0	313 / 137* , 175 , 113, 93	-17, -11, -11, -23	48.9	162.9	no standard
Benzoic acid-*O*-glucuronide [a,b)]	3.8	314.0	313 / 137* , 175 , 113, 93	-17, -11, -11, -23	55.9	186.3	no standard
Benzoic acid-*O*-glucuronide [a,b)]	4.6	314.0	313 / 137* , 175 , 113, 93	-17, -11, -11, -23	93.7	312.5	no standard
Benzoic acid-*O*-glucuronide [a,b)]	4.8	314.0	313 / 137* , 175 , 113, 93	-17, -11, -11, -23	44.9	149.6	no standard
Salicylic acid-*O*-glucuronide [a,b)]	7.8	314.1	313 / 137* , 93 , 75, 175, 113	-11, -23, -11, -11, -11	41.2	137.2	no standard
Salicylic acid-*O*-glucuronide [a,b)]	7.7	314.1	313 / 137* , 93 , 75, 175, 113	-11, -23, -11, -11, -11	46.5	155.0	no standard

Vanillin OR hydroxyphenylacetic acid-*O*-glucuronide [a,b)]	4.5	328.1	327 / 113* , 136 , 175, 151	-11, -23, -11, -23	111.6	372.1	no standard
Coumaric acid-*O*-glucuronide [a,b)]	9.1	328.1	327 / 113* , 107 , 175, 151	-17, -23, -11, -11	131.5	438.3	no standard
Coumaric acid-*O*-glucuronide [a,b)]	10.3	340.2	339 / 113* , 117 , 175, 163	-11, -23, -11, -17	62.9	209.7	no standard
(iso)vanillic acid-*O*-glucuronide [a,b)]	4.0	344.0	339 / 113* , 117 , 175, 163	-11, -23, -11, -17	85.4	284.6	no standard
(iso)vanillic acid-*O*-glucuronide [a,b)]	4.5	344.0	343 / 113* , 175 , 167, 152, 108	-17, -17, -17, -23, -23	102.6	341.9	no standard
Hippuric acid-*O*-glucuronide [a,b)]	4.9	355.2	354 / 113* , 134 , 175, 178	-11, -23, -11, -17	56.5	188.3	no standard
Caffeic acid-*O*-glucuronide [a,b)]	6.1	356.2	355 / 113* , 135 , 175, 179	-17, -23, -11, -17	109.2	364.2	no standard
Syringaldehyde OR Homovanillic acid OR dihydrocaffeic-*O*-glucuronide [a,b)]	5.1	358.2	357 / 113* , 175 , 181, 166, 123	-11, -11, -17, -23, -23	84.1	280.2	no standard
Syringaldehyde OR Homovanillic acid OR dihydroxycaffeic acid-*O*-glucuronide [a,b)]	3.9	358.2	357 / 113* , 175 , 181, 166, 123	-11, -11, -17, -23, -23	70.5	235.0	no standard
Dihydroferulic acid-*O*-glucuronide [a,b)]	5.1	372.3	371 / 113* , 195 , 85, 175	-17, -20, -23, -17	78.1	260.5	0.990
Syringic acid-*O*-glucuronide [a,b)]	7.5	374.2	373 / 113* , 175 , 197, 153	-17, -11, -11, -17	74.8	249.4	no standard
Syringic acid-*O*-glucuronide [a,b)]	9.0	374.2	373 / 113* , 175 , 197, 153	-17, -11, -11, -17	77.6	258.6	no standard

*sMRM transition used for quantification; [a)] site of conjugation could not be ascertained as identification was based on known transitions where pure standards for isomers were not available or separation of isomers was chromatographically not possible; [b)]tentatively identified compound which could have different isomeric configuration. Abbreviations: LOD, limit of detection (Signal/Noise = 3); LOQ, limit of quantification (Signal/Noise = 10); MW, molecular weight; RT, Retention time; sMRM, scheduled multiple reaction monitoring; R^2, linear regression coefficient of standard curve; no standard, no analytical standard was available to make standard curve

Supporting Information Table 5 – Urinary excretion rate per hour of non oat bran-derived phenolic compounds after intake of 60g oat bran or a control meal in six healthy men (nmol/h)

Non-metabolite	RT (min)	Meal	-12-0h	0-2h	2-4h	4-6h	6-8h	8-12h	12-24h	24-28h	28-32h	32-36h	36-48h	Inter-vention	Time	Inter-action
								(nmol/h)								
3,4-dihydrocaffeic acid	6.4	Oats	8±5	11±2	ND	54±11	164±31	60±28	15±4	6±1	81±20	63±26	18±5	0.2	****	0.7
		CON	10±3	10±4	9±4	50±11	138±23	47±16	20±5	6±3	100±17	44±13	13±4			
4-hydroxybenzaldehyde	5.9	Oats	9±3	43±12	29±6	37±11	48±9	23±8	8±3	24±7	30±9	18±6	12±3	0.5	****	0.2
		CON	4±2	11±4	24±9	17±6	35±6	16±5	12±5	15±7	30±10	21±5	8±3			
4-hydroxyphenylacetic acid	6.5	Oats	2944±997	7303±888	3598±538	4737±1289	9307±1394	3870±1227	2667±686	3229±754	4045±1013	3609±1237	3379±533	0.2	****	0.5
		CON	2658±622	5407±884	5961±2151	4948±859	9741±1885	4846±1390	3459±1119	4297±1125	4778±1278	6608±3173	2256±698			
3-hydroxyphenylacetic acid	6.3	Oats	21867±6765	63086±16587	29542±6008	39413±11293	72562±8932	26418±7768	23593±7204	39232±10190	39862±8965	26007±8449	30693±5066	0.3	****	0.1
		CON	20461±4935	44887±8620	47366±13805	27549±6555	63817±5442	23306±2171	27629±7689	25976±8532	33403±10462	38274±13067	15391±4559			
Dihydroisoferulic acid	7.5	Oats	15±7	36±23	23±8	22±11	42±4	7±3	20±5	28±18	25±12	16±7	19±6	0.2	*	0.6
		CON	7±3	10±3	18±16	53±	27±18	7±2	9±2	18±7	16±6	13±7	6±2			
Isoferulic acid	8.0	Oats	17±5	99±59	96±29	58±12	148±46	22±	7±1	13±7	61±20	47±22	9±2	0.2	***	0.5
		CON	15±3	20±7	24±16	24±	130±22	41±12	20±1	23±1	41±19	36±12	6±			
o-coumaric acid	8.4	Oats	2±	ND	ND	11±	17±10	ND	1±0	1±0	26±14	3±	0±	0.6	*	0.4
		CON	0±	4±3	3±3	9±6	39±3	5±	1±0	1±	22±9	2±1	0±			
Salicylic acid	8.1	Oats	67±59	171±64	143±54	77±21	222±120	39±19	17±5	95±66	103±36	109±82	30±14	**	0.1	0.2
		CON	6±2	17±7	39±17	32±12	43±7	24±11	11±5	20±9	27±8	24±8	9±3			
Syringaldehyde	7.5	Oats	0±0	0±0	0±0	1±0	0±0	ND	0±	0±	0±0	ND	0±0	0.2	**	0.4
		CON	0±0	0±0	0±	0±	0±0	0±0	0±0	0±	0±	1±	0±0			
Vanillin	6.9	Oats	3±1	11±3	13±3	18±2	24±2	9±4	4±1	8±3	17±3	8±2	4±1	0.2	****	0.3
		CON	2±0	3±1	10±3	18±6	19±2	4±1	4±1	5±1	20±2	7±1	3±1			
4-hydroxybenzoic acid	4.9	Oats	75±22	337±83	182±40	224±31	340±41	164±50	146±67	208±45	226±56	147±44	185±58	0.5	****	0.7
		CON	53±10	256±123	211±86	204±99	343±118	182±64	208±113	165±64	162±55	171±49	85±42			
Caffeic acid	6.0	Oats	34±8	119±29	91±26	111±19	161±18	80±35	53±13	73±18	81±18	62±20	62±9	0.1	****	0.4
		CON	30±4	61±16	87±24	67±15	128±21	60±7	47±15	46±18	78±30	80±23	33±10			
Gallic acid	1.7	Oats	1±1	1±0	2±1	2±1	2±1	3±2	1±1	2±1	1±0	2±1	1±1	0.8	0.8	0.8

		CON	0±0	1±1	2±2	2±1	1±0	0±0	0±0	1±0	1±0	5±4	0±0			
Hippuric acid	5.7	Oats	2772±703	9454±2193	7027±2028	7653±2036	12773±1494	5060±2449	4158±1001	5857±1432	6817±1545	5006±1627	4930±683	0.1	****	0.4
		CON	2403±339	5052±1396	7131±1944	5439±1199	10688±1668	4905±592	3905±1209	3593±1437	6311±2403	6525±1869	2696±853			
Homovanillic acid	6.2	Oats	251±64	894±261	584±76	726±164	1600±698	437±132	284±47	535±97	553±104	320±78	399±80	0.5	****	0.8
		CON	357±102	995±393	1116±189	1098±599	1616±309	657±316	352±81	959±384	1409±771	943±475	361±145			
Protocatechuic acid OR 3,5-dihydroxybenzoic acid	3.7	Oats	8±2	24±5	11±2	14±5	22±3	10±3	12±4	16±3	21±6	14±4	13±2	0.7	0.5	0.1
		CON	7±2	14±3	14±3	13±2	26±3	13±2	14±4	18±3	26±2	20±4	9±2			
Salicylic acid-*O*-sulfate	3.2	Oats	574±202	1619±473	689±213	1180±435	1224±269	1005±343	828±336	999±263	1094±362	563±231	802±281	0.5	*	*
		CON	488±190	804±242	903±434	640±208	944±207	675±156	775±147	1041±296	1107±435	1029±451	442±141			
Dihydroxybenzoic acid-*O*-sulfate	2.7	Oats	11±4	19±7	5±1	15±8	14±3	7±2	19±7	8±1	10±3	6±1**	14±4	0.1	0.3	**
		CON	9±2	13±3	9±4	7±2	18±5	21±8	14±3	8±2	13±4	26±13	7±2			
Coumaric acid-*O*-sulfate	9.0	Oats	27±6	30±10	11±2	21±5	35±10	20±4	22±7	14±4	18±8	11±4	13±2	0.3	0.5	0.2
		CON	8±1	17±7	17±6	13±2	26±4	128±110	11±2	18±6	29±18	70±34	59±39			
(iso)vanillic acid-*O*-sulfate	4.2	Oats	27±9	153±35	61±13	2956±767	3392±823	245±90	65±25	36±4	2658±490	168±45	61±11	0.7	****	0.9
		CON	32±9	27±7	42±13	2623±448	3272±883	217±53	65±19	28±7	2979±684	240±65	70±22			
Hippuric acid-*O*-sulfate	3.4	Oats	120±55	310±109	89±29	139±70	160±29	61±13	72±20	105±26	73±20	52±12	101±28	0.9	***	0.1
		CON	106±38	180±51	137±53	68±19	109±28	54±16	93±37	90±38	68±29	182±128	69±20			
Hippuric acid-*O*-sulfate	3.1	Oats	4±1	9±3	3±1	5±2	6±1	3±1	6±3	5±1	4±2	3±1	5±2	0.4	0.5	0.6
		CON	12±9	9±3	4±2	5±2	7±3	5±2	6±2	5±1	4±1	7±3	3±1			
Hippuric acid-*O*-sulfate	3.8	Oats	91±44	216±94	147±76	171±78	213±93	179±98	143±64	174±70	215±94	86±55	183±89	*	0.1	0.3
		CON	59±37	156±104	141±78	127±85	166±92	128±82	112±58	185±102	173±87	202±103	118±78			
Homovanillic acid OR dihydroxyhydroycinamic acid-*O*-sulfate	4.6	Oats	168±19	350±68	113±22	179±49	260±40	161±35	184±52	236±18	212±39	162±25	206±37	0.3	**	0.2
		CON	158±35	278±38	218±63	154±25	281±40	185±28	231±48	199±29	216±60	322±163	128±27			
Homovanillic acid34dihydrocaffeic-*O*-sulfate	4.9	Oats	115±35	152±49	32±9	117±29	171±45	172±88	88±25	60±11	101±31	191±81	172±20	**	***	0.3
		CON	165±43	101±31	41±11	56±20	91±23	115±40	125±31	37±4	43±14	190±65	74±34			
Hydroxybenzaledhyde-*O*-glucuronide	8.3	Oats	65±52	186±137	151±134	137±102	156±95	168±154	74±39	103±36	143±84	70±31	211±136	0.5	0.8	0.5
		CON	48±33	116±78	194±166	151±79	104±71	68±37	104±44	127±36	142±31	145±79	56±13			
Benzoic acid-*O*-glucuronide	2.7	Oats	11±3	35±10	12±5	18±8	18±4	12±3	23±10	21±9	19±7	13±4	17±6	0.3	*	0.5
		CON	9±5	18±8	18±12	13±6	20±10	11±6	15±6	17±6	17±6	16±8	6±2			

Benzoic acid-*O*-glucuronide	3.8	Oats	11±2	35±7	15±3	18±7	24±5	11±3	14±5	15±4	14±4	13±3	13±3	0.8	****	0.1
		CON	7±2	16±4	15±5	10±2	19±3	12±2	13±2	11±3	12±3	16±7	8±2			
Benzoic acid-*O*-glucuronide	4.6	Oats	3±1	7±2	6±1	8±3	14±5	3±1	3±1	4±1	5±1	4±1	3±0	0.3	****	0.3
		CON	2±1	5±1	6±2	ND	7±1	3±1	3±0	3±0	4±1	5±2	3±1			
Benzoic acid-*O*-glucuronide	4.8	Oats	2±1	ND**	ND	ND	ND	ND	1±	ND	ND	ND	2±1	0.1	****	**
		CON	2±0	2±1	ND	ND	ND	3±0	2±0	2±1	ND	6±2	1±1			
Salicylic acid-*O*-glucuronide	7.8	Oats	2±1	5±1	ND	5±3	12±	2±1	3±1	2±1	2±	1±	3±1	0.4	***	0.8
		CON	3±1	7±3	7±6	6±2	11±5	5±2	3±1	4±0	7±3	4±2	2±1			
Salicylic acid-*O*-glucuronide	7.7	Oats	2±1	7±1	ND	11±	5±2	2±1	3±2	3±1	3±	1±	3±2	0.9	***	0.9
		CON	3±2	8±4	13±13	7±3	14±10	5±2	3±1	4±1	5±1	5±2	2±1			
Vanillin OR hydroxyphenylacetic acid-*O*-glucuronide	4.5	Oats	26±9	68±14	32±6	44±14	71±11	27±7	30±8	34±7	37±8	32±10	34±7	0.3	***	0.2
		CON	18±3	39±5	49±12	31±4	65±12	35±5	39±5	27±6	32±6	58±38	17±7			
Coumaric acid-*O*-glucuronide	9.1	Oats	19±8	55±24	22±2	29±8	35±3	26±11	24±5	17±5	21±1	17±9	15±3	*	0.5	0.2
		CON	14±4	37±18	33±17	24±7	29±4	96±70	46±32	23±6	36±23	120±80	60±43			
Coumaric acid-*O*-glucuronide	10.3	Oats	43±32	58±21	17±5	21±9	23±6	8±3	11±3	10±4	8±2	11±4	15±3	1	0.2	0.5
		CON	150±137	29±12	23±5	23±11	35±16	47±36	115±102	17±10	20±11	18±8	9±3			
(iso)vanillic acid-*O*-glucuronide	4.0	Oats	35±12	127±38	93±12	1720±453	3518±501	672±248	143±38	44±8	1811±500	676±222	149±31	*	****	0.9
		CON	49±14	46±11	52±13	1466±284	3110±367	591±152	150±25	32±8	1628±449	528±114	163±62			
(iso)vanillic acid-*O*-glucuronide	4.5	Oats	165±52	677±159	367±54	7116±1491	10045±1653	1591±596	382±118	214±30	6104±1656	1568±521	478±96	0.2	****	1
		CON	187±44	237±68	219±52	6227±994	9168±1313	1512±396	450±63	147±33	5892±1604	1459±320	424±130			
Hippuric acid-*O*-glucuronide	4.9	Oats	54±21	144±48	58±20	84±38	103±25	47±20	50±22	65±24	84±30	64±22	50±19	0.8	***	0.2
		CON	31±14	54±27	71±43	38±20	96±40	44±20	38±14	48±18	63±22	56±30	30±14			
Caffeic acid-*O*-glucuronide	6.1	Oats	16±7	57±17	19±	21±3	25±9	5±2	8±2	13±3	7±0	12±4	23±2	0.7	0.2	0.1
		CON	33±24	25±4	13±1	16±5	24±5	19±7	40±22	11±5	22±16	18±7	20±9			
Syringaldehyde OR Homovanillic acid OR dihydrocaffeic-*O*-glucuronide	5.1	Oats	13±3	45±11	22±4	31±6	47±7	23±6	24±5	32±7	30±5	24±6	26±4	0.1	****	0.4
		CON	16±4	29±5	29±7	24±4	42±4	25±4	28±4	26±4	30±4	34±13	17±5			
Syringaldehyde OR Homovanillic acid OR dihydroxycaffeic acid-*O*-glucuronide	3.9	Oats	7±1	21±2	12±2	14±3	21±3	12±4	9±2	12±2	14±3	11±3	9±2	0.1	****	0.4

		CON	5±1	11±3	13±3	10±2	17±1	9±2	8±1	8±2	13±2	12±3	6±1			
Dihydroferulic acid-*O*-glucuronide	5.1	Oats	6±2	17±4	8±2	10±4	21±5	5±1	7±2	7±2	16±5	9±2	7±2	0.5	***	0.4
		CON	5±1	7±1	8±3	17±7	38±19	11±3	7±1	6±1	15±5	11±3	5±1			
Syringic acid-*O*-glucuronide	7.5	Oats	249±66	700±75	268±41	399±60	535±77	276±79	357±55	407±72	327±80	319±101	415±56	0.5	***	0.1
		CON	258±38	426±44	333±67	276±28	498±46	294±25	390±37	316±49	400±73	479±157	258±65			
Syringic acid-*O*-glucuronide	9.0	Oats	56±29	183±47	126±66	194±71	277±77	175±82	132±31	178±53	146±55	141±54	145±20	*	****	0.4
		CON	88±31	171±54	140±34	150±47	259±60	175±59	138±31	144±44	213±67	197±55	112±44			

Data are reported as mean ± SEM and were analysed by two-factor repeated measurement linear mixed model with time and treatment as the two factors. When the interaction effect was significant, post-hoc analyses with Tukey-Kramer adjustment were performed. If excretion was ND, the concentration was replaced with the limit of detection for statistical analysis. Model P values in the three far right columns and post-hoc P values next to the excretion rates are indicated as follow: * P < 0.05; ** P <0.01; *** P<0.001; **** P<0.0001. ND; not detected; CON control.

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